



Methylisothiazolinone toxicity on the freshwater keystone species *Daphnia pulex*

Margot Wagner-Deyries

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THÈSE DE DOCTORAT DE

L'INSTITUT AGRO RENNES ANGERS

ECOLE DOCTORALE N° 600

Ecologie, Géosciences, Agronomie, Alimentation

Spécialité : *Ecologie et évolution*

Par

Margot WAGNER-DEYRIES

Toxicité de la méthylisothiazolinone sur l'espèce clé de voûte des écosystèmes d'eau douce *Daphnia pulex*

Methylisothiazolinone toxicity on the freshwater keystone species *Daphnia pulex*

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ABSTRACT

Methylisothiazolinone toxicity on the freshwater keystone species *Daphnia pulex*

Keyword: Multigenerational effect, biocide, intraspecific variability, evolutionary ecotoxicology, transcriptome

Isothiazolinones are a family of organic molecules used as broad-spectrum biocides in a wide variety of consumer and industrial applications. Although their presence in the environment has been detected, especially in soils and freshwater, reports by the manufacturers deemed the quantities involved harmless for the ecosystem. However, given the daily amounts released into the environment over decades, concerns have been raised about their impact on non-target species, particularly with respect to potential effects over multiple generations, which cannot be examined under standard ecotoxicity test conditions. This work aims to provide some answers by studying the effects of the most innocuous of these molecules, methylisothiazolinone (MIT). MIT acute toxicity was assessed on *Daphnia pulex* (Cladocera), an iconic ecotoxicology and evolutionary model species of aquatic invertebrates.

MIT exposure had both lethal and sublethal effects on daphnia, and affected their fitness. Furthermore, these effects developed over generations in the case of continuous multigenerational exposure, but not in the case of parental or grandparental exposure alone. In addition, the tests revealed significant intraspecific variability in the tolerance to MIT contamination, both in the short term (48 hours) and longer term (chronic toxicity, sublethal concentrations). These effects were further investigated by transcriptomic expression analysis (RNAseq). Both intraspecific variability and multigenerational changes in the responses within one species call for the inclusion of evolutionary processes in risk assessments and the reconsideration of standard ecotoxicity test interpretations.

RÉSUMÉ FRANÇAIS

Toxicité de la méthylisothiazolinone sur *Daphnia pulex*, espèce clé de voûte des écosystèmes d'eau douce

Mots clés : Effet multigénération, biocide, variabilité intraspécifique, écotoxicologie évolutive, transcriptome

Les isothiazolinones sont des molécules organiques utilisées comme biocides à large spectre dans une grande variété d'applications industrielles et de consommation. Bien que détectées dans les sols et les eaux douces, les quantités concernées sont considérées inoffensives pour l'écosystème. Toutefois, compte tenu des quantités libérées dans l'environnement depuis les dernières décennies, des inquiétudes ont été exprimées quant à leur impact sur les processus d'évolution des espèces non cibles, qui ne sont pas examinés dans les tests de toxicité standard. Ce travail visait à étudier en particulier les effets de la plus inoffensive de ces molécules, la méthylisothiazolinone (MIT), sur *Daphnia pulex*, invertébré aquatique emblématique en écotoxicologie et en biologie évolutive. L'exposition à la MIT a eu des effets létaux et sublétaux sur les daphnies et a affecté leur fitness. De plus, ces effets se sont modifiés au cours des générations dans le cas d'une exposition multigénérationnelle continue, mais pas dans le cas d'une exposition uniquement grand-maternelle. En outre, les tests ont révélé une variabilité intraspécifique significative de la tolérance, aussi bien à court terme (toxicité aiguë, 48 heures) qu'à plus long terme (toxicité chronique, concentrations sublétales). Ces effets ont été étudiés plus en détail par analyse d'expression transcriptomique (RNAseq). La variabilité intraspécifique et les changements dans les réponses générationnelles appellent à l'inclusion des processus évolutifs dans les procédures d'évaluation de risque et au réexamen des interprétations des tests d'écotoxicité standard.

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ABBREVIATIONS

DEB – Dynamic Energy Budgets

DEG – Differentially Expressed Gene

DET – Differentially Expressed Transcript

D. pulex – *Daphnia pulex*

ERA – Environmental Risk Assessment

GO – Gene Ontology

GUTS-IT – General Unified Threshold model for Survival – Individual Tolerance

HM – High MIT concentration (50 µg/L)

LC50 – Median Lethal Concentration

LM – Low MIT concentration (0.8 µg/L)

MG – multigenerational

NA – missing data

ncRNA – non-coding Ribonucleic acid

NOEC – No Observed Effect Concentration

NS – not significant

TG – transgenerational

TKTD – Toxicokinetics-Toxicodynamics

UPLC-MS/MS – UltraPerformance Liquid Chromatography - tandem mass spectrometry

LIST OF COMMUNICATIONS

Published papers

Wagner-Deyriès, M., Varignier, L., Revel, M., Delhay, T., Rondeau, D., Coutellec, M.-A., & McCairns, R. J. S. (2023). Variation of Tolerance to Isothiazolinones Among *Daphnia pulex* Clones. *Environmental Toxicology and Chemistry*, 42(4), 805-814.
<https://doi.org/10.1002/etc.5564>

Posters

Variability of Tolerance to Methylisothiazolinone Among Independent Lines of *Daphnia pulex*, SETAC 2022 - Copenhagen

Oral communications

Variability of tolerance to methylisothiazolinone among independent lines of *Daphnia pulex* over four generations. (oral presentation) ISEPEP 2022 – Rennes
Variability of tolerance to methylisothiazolinone among independent lines of *Daphnia pulex*. (oral presentation) Journées scientifiques EGAAL 2022 – Rennes
Variabilité de la tolérance de lignées de daphnies aux isothiazolinones (Flash talk) GDR ECOTOX (INRAE) 2020 – Online

ATTENDED COURSES

Bioinformatics Pipeline Development with Nextflow, 22-25/11/2021, ECSEQ Bioinformatics, Online
Ecological and evolutionary studies on DNA Methylation in plants and animals, 15-18/11/2021, Physalia, Online
Membre comité organisation Journées scientifiques ED EGAAL 2021, 04/10/2021, Online
Speaking/pitching in english, 8-10/03/2021, Online
Communiquer sur sa recherche : communiqués de presse, interviews, 02/02/2021, URFIST Strasbourg
Introduction à l'éthique, 18/07/2020, Nantes
Papyrus – Parcours d'accompagnement Publier & Communiquer en science 2020 Session n° 1, 17/01/2020 - 13/03/2020, INRAE Rennes
Journées thématiques Modélisation TKTD du GDR Ecotoxicologie Aquatique, 20-21/11/2019, Université de Bordeaux
Journées thématiques OMICS du GDR Ecotoxicologie Aquatique, 18-19/11/2019, Université de Bordeaux

SYNTHÈSE DES TRAVAUX (FRANÇAIS)

INTRODUCTION – Étude des effets d'un xénobiotique dans un cadre d'écotoxicologie évolutive

Les travaux présentés dans cette thèse s'adressent à la compréhension des effets de la pollution chimique sur la biodiversité, le concept de biodiversité étant défini par la Convention sur la diversité biologique¹ par la « variabilité des organismes vivants de toute origine y compris, entre autres, les écosystèmes terrestres, marins et autres écosystèmes aquatiques et les complexes écologiques dont ils font partie ; cela comprend la diversité au sein des espèces et entre espèces ainsi que celle des écosystèmes. ». L'intégrité des écosystèmes actuels dépendent ainsi de cette diversité biologique. Or l'origine de la crise de la biodiversité actuelle², qui se traduit par le déclin du nombre de formes de vie, peut être résumée par cinq facteurs : la modification et destruction des habitats, la surexploitation des ressources naturelles, le changement climatique, les invasions d'espèces exotiques et la pollution³. Les travaux de cette thèse se focalisent sur les conséquences de la pollution chimique d'origine anthropique. Ces produits et molécules (engrais, pesticides, métaux lourds, détergents, cosmétiques, etc.) utilisés à des fins spécifiques peuvent impacter des organismes non ciblés initialement, en particulier lorsqu'ils sont retrouvés dans les écosystèmes naturels : c'est l'objet d'étude de l'écotoxicologie.

Le premier type de conséquences écologiques de la pollution chimique attendues est la disparition (mort ou migration) des individus ou espèces les plus sensibles. C'est le principe de la bioindication⁴ qui repose sur la présence/absence des espèces les plus sensibles pour définir la qualité d'un milieu. Les contaminants chimiques ont aussi des effets notoires sublétaux sur la physiologie des organismes, ces effets se répercutant potentiellement sur leur durée de vie, leur développement, leur fertilité ou encore leur comportement^{5,6}. Parmi ces effets sublétaux, les perturbateurs endocriniens ont reçu une attention particulière croissante depuis les deux dernières décennies. Si ces substances qui interrompent le bon fonctionnement du système hormonal sont particulièrement connues pour

¹Convention sur la diversité biologique, 1992

²Rapport de l'IPBES, 2019

³Sigmund *et al.*, 2023, *Global Change Biology*, 29, 3240–3255

⁴Verneaux & Tuffery, 1967, *Annales Scientifiques de l'Université de Besançon, Zoologie*, 3, 79-90

⁵Muyssen *et al.*, 2010, *Ecotoxicology and Environmental Safety*, 73, 5, 735-42

⁶Ashauer & Jager, 2018, *Environmental Science: Processes & Impacts*, 20,1,48-57

leur effet néfaste sur la reproduction humaine, leur impact sur les espèces sauvages est lui aussi de plus en plus reconnu⁷.

Les écosystèmes aquatiques, en particulier d'eau douce, sont largement concernés par des pressions de pollution⁸. Il est donc nécessaire de mieux comprendre les effets de ces pressions sur les écosystèmes, *a fortiori* à long terme. L'étude des effets directs d'une exposition à la pollution chimique peut avoir lieu à l'échelle moléculaire ou cellulaire avec une analyse des répercussions sur le développement, la croissance ou le comportement^{6,9}, mais les conséquences peuvent également être de nature indirecte. En effet, certains organismes vont consacrer une part non négligeable de leur budget énergétique dans le maintien de leur intégrité sous pression chimique au lieu de mobiliser cette énergie pour la reproduction, la croissance ou leur maintenance¹⁰. Aussi, face à un stress non léthal, la plupart des organismes vont avoir une réponse plastique leur permettant d'atténuer les effets néfastes d'une contamination¹¹, voire augmenter leurs performances par effet hormétique¹². En plus de la tolérance intrinsèque des individus, le stress chimique peut agir au niveau de la transmission d'une génération à une autre, via une modification d'allocation d'énergie¹⁰, une perturbation du système endocrinien responsable du développement¹³, ou des marques épigénétiques transmissibles¹⁴. La transmission peut avoir lieu en présence du polluant (effet intergénérationnel) ou même en absence de contamination directe (effet transgénérationnel) dans le cas d'individus jamais exposés au facteur de stress d'origine mais subissant les conséquences d'une exposition ancestrale¹⁵. A l'échelle d'une population, dont les individus présentent des niveaux de tolérance différents pour un contaminant donné, l'arrivée du produit dans le milieu va favoriser les individus les plus tolérants -qui auront une meilleur fitness (plus grande descendance fertile)- en exerçant une pression sur les moins tolérants. Si l'origine de la tolérance est génétique, le phénomène va contribuer à réduire la diversité phénotypique et génotypique de la population au cours des générations. Cependant, en théorie, une diversité génétique réduite est synonyme d'un potentiel évolutif moindre, c'est-à-dire que cette faible diversité constitue une entrave à la capacité de la population à s'adapter à un changement dans le milieu, tel que l'entrée d'un nouveau contaminant ou une hausse de la température¹⁶.

⁷Marlatt *et al.*, 2022, *Environmental research*, 208, 112584.

⁸Malaj *et al.*, 2014, *Proceedings of the National Academy of Sciences*, 111, 26, 9549-9554

⁹Lebrun *et al.*, 2021, *Chemosphere*, 277, 130277

¹⁰Jeon *et al.*, 2013, *Ecotoxicology and Environmental Safety*, 98, 28-35

¹¹Ashauer *et al.*, 2015, *Environmental Science & Technology*, 49,16, 10136-46

¹²Calabrese, 2004, *EMBO reports*, 5, S1, S37-S40

¹³Zhang *et al.*, 2008, *Environmental Science & Technology*, 42, 21, 8133-39

¹⁴Vandeghehuchte & Janssen, 2011, *Ecotoxicology*, 20, 3, 607-24

¹⁵Coutellec & Barata, 2013, *Ecotoxicology*, 22, 5, 763-66

¹⁶Delnat *et al.*, 2022, *Environmental Pollution*, 308, 119654

Les travaux de cette thèse se situent ainsi à l'interface des échelles individu et population, négligée en écotoxicologie classique. Le but étant de mieux comprendre les effets d'une contamination chimique sur les traits liés à la fitness des organismes, et comment ils se transmettent d'une génération à l'autre. Pour aborder cette problématique, j'ai choisi de m'intéresser à l'impact de la contamination par la méthylisothiazolinone (MIT) sur une espèce de daphnie, en adoptant une approche expérimentale en laboratoire.

La MIT est une molécule organique (Figure viii, p.38) listée en France parmi les substances à surveiller dans les eaux de surface continentales¹⁷. Ses applications sont nombreuses, elle est en particulier utilisée comme conservateur grâce à son activité de biocide à spectre large dans une grande variété de produits d'usage courant, notamment les cosmétiques, les produits d'hygiène et les peintures murales¹⁸. De par ses utilisations multiples, ses rejets réguliers et sa dégradation incomplète dans les centrales de traitement des eaux usées¹⁹, la MIT peut être considérée comme pseudo-persistante dans les eaux environnementales. A ma connaissance, seules trois mesures de concentrations ont été menées dans des eaux de surface^{19,20,21}, ne donnant qu'une idée très approximative de la présence réelle de cette molécule, ce qui la classe en « polluant émergent ». Quelques valeurs de concentrations létales (CL50) pour la MIT existent déjà pour certaines espèces aquatiques (Tableau ii, p.39), mais peu d'effets sublétaux sont connus. Ces derniers comprennent des effets de retardement de régénération chez le têtard de xénope²² et la planaire²³, des lésions histologiques et de la génotoxicité chez la truite arc-en-ciel²⁴, et des retards de développement avec une perturbation du système thyroïdien chez le zebrafish²⁵.

Le modèle biologique choisi pour ce projet, la daphnie, est un organisme clé de voûte des systèmes lenti-ques²⁶. Ce crustacé zooplanctonique a en effet un rôle central dans son écosystème, à la fois source de nourriture et prédateur de nombreuses espèces. De plus, son mode de reproduction cyclique par parthénogénèse permet de mettre en place des lignées clonales en laboratoire relativement simplement et ainsi de contrôler les effets génotypiques dans des conditions expérimentales. Les daphnies sont ainsi des modèles biologiques reconnus et couramment utilisés en biologie, écologie et génétique quantitative²⁶. L'espèce *Daphnia pulex* en particulier a été choisie ici,

¹⁷Arrêté du 26 avril 2022 modifiant l'arrêté du 25 janvier 2010 établissant le programme de surveillance de l'état des eaux en application de l'article R. 212-22 du code de l'environnement

¹⁸Silva et al., 2020, *Molecules*, 25, 4,991

¹⁹Paijens et al., 2020, *Water, Air, & Soil Pollution*, 231, 5, 210

²⁰Baranowska & Wojciechowska, 2013, *Polish Journal of Environmental Studies*, 22, 6, 1609-25

²¹Nowak et al., 2020, *Chemosphere*, 254, 126723

²²Delos Santos et al., 2016, *Aquatic Toxicology* 181, 37-45

²³Van Huizen et al., 2017, *Aquatic Toxicology*, 191, 226-35

²⁴Capkin et al., 2017, *Chemosphere*, 187, 720-29

²⁵Lee et al., 2023, *Ecotoxicology and Environmental Safety*, 249, 114406

²⁶Miner et al., 2012, *Proceedings of the Royal Society B: Biological Sciences*, 279, 1735, 1873-82

car son génome a été le premier chez les puces d'eau à être séquencé et annoté²⁷, et qu'aucun test avec la MIT n'a été mené jusqu'ici avec cette espèce.

En utilisant *D. pulex* et la MIT comme modèles, j'ai donc tenté d'évaluer les effets toxiques d'une exposition chronique à un polluant émergent dans un contexte de biologie évolutive. Les questions qui ont guidé cette étude ont été : (1) Différentes lignées clonales ont-elles des réactions différentes à la contamination (estimation de l'effet du génotype) ? (2) Est-ce que la MIT provoque un stress à de faibles concentrations, proches des concentrations environnementales mesurées ? (3) Observe-t-on un changement au cours des générations de ces réactions (patron d'exposition multi- ou transgénérationnel) ? (4) Y a-t-il des mécanismes particulièrement impactés par la contamination à l'échelle du transcriptome (RNAseq) ?

CHAPITRE 1 - Variations de tolérance aux isothiazolinones chez *D. pulex*

Des tests de toxicité aiguë, c'est-à-dire la toxicité induite par l'administration d'une dose forte de produit toxique dans un laps de temps court, ont été menés sur 8 clones de daphnies issus de populations naturelles différentes ou bien de populations de laboratoire (provenances voir Tableau S1-1, p.114). Chaque lignée clonale provenait d'une femelle qui s'est reproduit par parthénogenèse. Pour chaque lignée, une CL50 a été mesurée en appliquant le protocole standard 202 de l'OCDE²⁸ (50 répliques) pour deux biocides : la MIT seule et le mélange CMIT/MIT (3:1). En effet, le mélange de chlorométhyl- et méthyl-isothiazolinone (CMIT/MIT) était utilisé plus communément avant que la synthèse de la MIT seule – moins toxique – ne soit optimisée¹⁸. Aucune données de CL50 n'existait pour *D. pulex* pour aucun des deux biocides avant l'étude présentée ici²⁹. Les résultats ont été appuyés par le dosage des molécules cibles sur UPLC-MS/MS dont la méthode a été développée avec l'appui de la plateforme de métabolomique de l'IETR de Rennes.

Les résultats des tests de survie à 48h après le début de la contamination sont indiqués en Figure 1-3 (CMIT/MIT, p.55) et Figure 1-4 (MIT, p.56), sous la forme de (A) courbes de survie (modélisation TKTD-GUTS-IT) et (B) CL50 (calculées après GLMM) pour chaque clone séparément. Ces lignées clonales ont présenté de fortes variations dans leurs réponses, avec des CL50 allant de 0,10 à 1,84 mg/L pour le mélange CMIT/MIT, et de 0,68 à 2,84 mg/L pour la MIT seule. Ces fourchettes intraspécifiques de CL50 remettent en question l'utilisation de clones uniques de daphnies dans les tests d'écotoxicologie standards et la fiabilité des prédictions basées sur leurs résultats. Cette étude apporte de nouvelles preuves que l'évaluation du risque écologique des produits chimiques ne doit

²⁷Colbourne *et al.*, 2011, *Science* 331, 6017, 555-61

²⁸OECD, 2004, *OECD Guidelines for the Testing of Chemicals, Section 2*

²⁹Wagner-Deyriès *et al.*, 2023, *Environmental Toxicology and Chemistry*, 42, 4, 805-14

pas ignorer la diversité génotypique car ce n'est pas pertinent d'un point de vue écologique, et ne permet pas non plus une évaluation représentative de la diversité des effets néfastes potentiels.

Ces résultats m'ont aussi permis de préparer les conditions des tests sublétaux présentés dans les chapitres 2 et 3, en sélectionnant d'une part 6 clones susceptibles d'avoir une grande variation dans leurs réponses, et d'autre part en identifiant 2 doses supposées sublétales de MIT. Une dose inférieure à 1/100 de la CL50 la plus faible, d'après un facteur de sécurité qui est parfois pris pour définir des concentrations acceptables, ici 50 µg/L ; et une dose encore plus faible, correspondant à la concentration maximale de MIT relevée dans des eaux de surface, 0,8 µg/L. Ces limitations en nombre de clones et de concentrations testées étaient dues aux limitations techniques des tests simultanés. Ces tests ont été conduits sur plusieurs générations, afin d'identifier les effets d'une exposition chronique à la MIT, en observant les effets à l'échelle macrophénotypique (chapitre 2) et transcriptomique (chapitre 3).

CHAPITRE 2 - Effets multigénérationnels de doses légères de méthylisothiazolinone sur la fitness de clones de puces d'eau:

Les effets latents de faibles concentrations de la MIT sur la fitness de *D. pulex* ont été mesurés dans diverses configurations d'exposition chronique. Tous les tests ont été menés au long de 4 générations (de la parentale P à la F3), chaque daphnie ayant été surveillée de sa naissance à son 21ème jour. En plus des daphnies non exposées, 2 doses de MIT (0,8 et 50 µg/L) ont été testées, pour 6 lignées de daphnie, avec 10 répliques dans 10 béciers séparés. Au cours des générations, les daphnies exposées l'ont été soit en continue (exposition directe), soit seulement dans les premières générations (jours 1 à 21 de la P et jours 1 à 11 de la F1) avec un transfert en eau non contaminée avant que la génération F2 ne se développe dans les femelles F1, de sorte que les femelles F2 ont été exposées seulement au stade de cellules germinales, et les F3 n'ont jamais été exposées directement (exposition uniquement parentale ou ancestrale). Ce design devait permettre de mesurer d'éventuels effets multigénérationnels ou transgénérationnels.

La survie et la reproduction ont été relevées quotidiennement, puis ont été résumées sous un indice de fitness individuelle décrite dans la Formule 1 (p.64), similaire à la valeur reproductrice définie par Fisher³⁰, prenant en compte la survie, le nombre de petits produits par jour et le timing de reproduction. Les scores de fitness individuelles ont ensuite été comparés entre les différents

³⁰Fisher, 1999, *The Genetical Theory of Natural Selection: A Complete Variorum Edition*

traitements, ce qui a permis de constater des effets létaux et sublétaux dans les scénarios d'exposition directe, mais pas dans le cas des exposition parentale.

Si aucun effet n'a été relevé après une exposition à la MIT seulement à l'étape germinale (F2) ou parentale uniquement (F3), les deux doses de MIT testées de manière continue au cours des générations de daphnies ont provoqué des réponses différentes. La dose la plus faible (0,8 µg/L) n'a pas modifié la valeur médiane de fitness par rapport à celle des contrôles, cependant la distribution des valeurs était plus étalée, correspondant à un plus grand nombre d'individus qui ne se sont pas reproduit avant la fin du test (Figure 2-3, p.74). Cette tendance qui s'est accentuée de génération en génération semblait traduire une sensibilisation des daphnies à la MIT. Concernant l'effet multigénérationnel d'une dose plus forte (50 µg/L), la dynamique révélée était opposée, avec un effet fort et immédiat en F1, puis une récupération progressive des valeurs de fitness vers des valeurs similaires aux contrôles, dans un probable processus d'acclimatation. Dans le cas de chaque dose testée, toutes les lignées clonales n'ont pas répondu de la même manière, révélant des sensibilités variées (Figure 2-5, p.76). Cependant les clones les plus sensibles dans le chapitre 2 ne sont pas systématiquement les mêmes que ceux découverts dans le chapitre 1.

Ce deuxième chapitre a ainsi montré qu'une exposition chronique à des doses environnementales de MIT a eu des effets néfastes sur cette espèce non cible. De plus, l'importance de prendre en compte la variabilité intraspécifique dans l'évaluation de toxicité a aussi été rappelée, car l'intensité et la temporalité des effets observés dépendaient grandement du génotype.

Au 21ème jour des daphnies survivantes, par groupe de lignées et de générations, les crustacés ont été sacrifiés afin de faire une extraction d'ARN pour essayer de mettre en relation les réponses macro- et micro-phénotypique après une exposition à la MIT. Ces résultats sont présentés dans le chapitre 3.

CHAPITRE 3 - Aperçu des modifications moléculaires suite à une exposition à la MIT, à l'échelle du transcriptome :

L'ARN des daphnies adultes exposées ou non à la MIT durant leur vie a été séquencé, puis les données d'RNA-seq ont été assemblées avec une méthode hybride proposée par la suite TRINITY³¹ entre *de novo* et guidée par un génome de référence (*Daphnia pulex* KAP4³²) afin de capturer un maximum de variations entre lignées. Une analyse d'expression différentielle a ensuite permis d'identifier les gènes significativement sur- ou sous-exprimés chez les daphnies exposées au biocide,

³¹Grabherr *et al.*, 2011, *Nature Biotechnology*, 29, 7, 644-52

³²NCBI RefSeq assembly GCF_021134715.1, 2022

puis ces transcrits différentiellement exprimés ont été annotés sur le protéome de référence de *D. pulex*. Enfin une analyse d'enrichissement par annotation des termes GO (Gene Ontology) a été menée pour en déduire les principaux processus biologiques, fonctions moléculaires et composants cellulaires les plus affectés par l'exposition à la MIT.

Dans l'ensemble, les catégories suivantes étaient significativement enrichies : activités hydrolase/protéolyse/peptidases, division cellulaire, synthèse d'acides aminés, transport d'ions, mort cellulaire, activité antioxydante et de manière plus spécifique le métabolisme d'ecdystéroïdes. Les résultats n'étaient pas homogènes entre les différentes lignées, mais ces termes enrichis étaient récurrents et correspondent de plus à une réponse générale face à un stress chimique, excepté pour la régulation d'ecdystéroïdes qui sont spécifiques au système hormonal des arthropodes. Étant donné le mode d'action des isothiazolinones qui repose sur leur haute réactivité avec les groupements thiols - entravant par là les fonctions de respiration cellulaire et de synthèse d'ATP notamment³³ - la réponse généraliste n'est pas étonnante. En observant les résultats en nombre de transcrits différentiels pour l'ensemble des clones et des conditions, aucun patron temporel particulier n'a pu être mis en évidence (transcrits communs à une génération chez tous les clones par exemple). En effet la majorité des transcrits étaient spécifiques aux traitements et aux clones, cependant en regroupant tous les transcrits différentiels partagés par au moins 5 des 6 clones pour toutes les conditions, 26 transcrits ressortaient dont 25 sont des ARNs non codants (lncRNA) (Tableau 3-1, p.99).

Ces lncRNA sont très nombreux dans le génome³⁴ et sont identifiés comme impliqués dans des maladies, en particulier des cancers chez les humains³⁵. La liste de leurs fonctions est longue et comprend de nombreuses fonctions de régulation d'expression. Chez les arthropodes, des lncRNA ont aussi été associés à des réponses enzymatiques de défense contre un stress chimique³⁶, et à des mécanismes de développement via la régulation de la vitellogénèse³⁷. Ce type de régulateur peut donc avoir un lien direct avec une modification du phénotype via leur impact sur les capacités de reproduction par exemple, et donc avec une modification de la fitness. Ce type de régulation après une exposition des daphnies à la MIT ne semble donc pas surprenante ; on remarque tout de même que tous les types d'exposition, y compris transgénérationnelle, semblaient globalement provoquer des réponses similaires au niveau du transcriptome.

³³Williams, 2007, *PowerPlant Chemistry*, 9, 1, 9

³⁴Djebali *et al.*, 2012, *PLoS One*, 7, 1, e28213

³⁵Esteller, 2011, *F1000 biology reports*, 3

³⁶Valenzuela-Miranda *et al.*, 2017, *Agri Gene*, 4, 1-7

³⁷Thepsuwan *et al.*, 2021, *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 261, 111045

La régulation épigénétique semble ainsi jouer un rôle clé dans la réponse moléculaire à la toxicité de la MIT, rôle partagé dans toutes les lignées clonales à toutes les générations, sous la forme de lncRNA qui ont eux-même des fonctions de régulation (non déterminées pour l'heure). Mais en ce qui concerne les ARN codants pour des protéines, la majorité des réponses semblaient spécifiques aux clones, tout en renvoyant néanmoins - par redondance génétique fonctionnelle – à des processus communs. Aucun lien n'a cependant pu être établi avec les réponses macro-phénotypiques présentées dans le chapitre 2.

CONCLUSION

Dans un contexte d'écotoxicologie évolutive, les travaux présentés ici ont répondu à plusieurs objectifs par une approche empirique en utilisant *D. pulex* comme espèce modèle. Certains points des résultats sont discutés ci-dessous.

Concernant les effets chroniques relevés sur la fitness individuelle des adultes, bien que l'on aurait pu anticiper l'émergence d'un effet défavorable sur la condition physique au fil des générations, il est à noter que l'adaptabilité transgénérationnelle plastique qui a été observée est quelque peu surprenante. Dans la première génération F1, même avec l'utilisation de doses supposées sublétales, la grande majorité des daphnies exposées dès leur premier stade de développement à une concentration de 50 µg/L n'ont pas survécu suffisamment longtemps pour parvenir à la phase de reproduction. De ce fait, l'application d'un facteur de sécurité équivalent au centième de la valeur de CL50 ne s'avère pas valide dans le contexte de la relation entre MIT et daphnie. Il convient également de mentionner que l'extrapolation de valeurs de concentration présumées "non néfastes" à long terme à partir de tests de toxicité aiguë est une pratique remise en question depuis plusieurs décennies^{38,39,40,41,42}. Cette remise en question est en partie due à la disparité des mécanismes sous-jacents à la toxicité aiguë et à la toxicité chronique.

En outre, les valeurs de fitness des daphnies exposées à 0,8 µg/L en F3 semblaient converger avec celles exposées à 50 µg/L. Cette constatation soulève des préoccupations quant aux risques potentiellement sous-évalués associés à la présence du biocide dans les eaux de surface, même à de faibles concentrations⁴³. Cela accentue la nécessité d'instaurer de manière plus systématique des

³⁸Crane & Newman, 2000, *Environmental Toxicology and Chemistry* 19, 2, 516-19

³⁹Barata *et al.*, 2002, *Aquatic Toxicology*, 60, 1, 85-99

⁴⁰Medina *et al.*, 2007, *Chemosphere*, 67, 11, 2105-14

⁴¹Coutellec & Barata, 2011, *Ecotoxicology*, 20, 3, 493-96

⁴²Côte *et al.*, 2015, *Environmental Pollution*, 205, 209-17

⁴³Kresmann *et al.*, 2018, *Science of The Total Environment* 625, 900-908

essais multigénérationnels en écotoxicologie. Il est à noter que quatre protocoles d'essais multigénérationnels standards ont déjà reçu l'approbation des agences environnementales européennes et américaines. Ces protocoles utilisent des modèles de poissons d'eau douce⁴⁴, le médaka⁴⁵, la caille du Japon⁴⁶ et le chironome⁴⁷. De plus, deux protocoles similaires sont actuellement en évaluation, dont l'un s'appuie sur la daphnie en tant que modèle biologique⁴⁸.

Ces tests multigénérationnels s'avèrent particulièrement appropriés pour l'exploration des effets endocriniens, ce qui renvoie à la dimension moléculaire de la présente étude. En effet, si l'analyse des données RNAseq présentées dans ces travaux demeure superficielle, elle met néanmoins en évidence des fonctions d'intérêt. Des pistes ont été identifiées concernant les mécanismes moléculaires associés à un stress chimique global, ainsi que des signes de perturbation endocrinienne. Ces découvertes corroborent les observations réalisées sur la truite arc-en-ciel²⁴ et le zebrafish²⁵.

Néanmoins, il n'est pas possible d'affirmer ou de réfuter l'altération de ces fonctions sans disposer de données à l'échelle cellulaire, obtenues par des méthodes telles que la protéomique ou la métabolomique, voire en divisant l'analyse par tissus ou organes. Par exemple, la dissociation des œufs et des embryons par dissection avant l'extraction permettrait de confirmer si les perturbations des hormones de croissance observées résultent bien de l'effet du biocide sur l'organisme adulte plutôt que de l'effet sur la croissance des embryons.

De plus, il convient de souligner que ces résultats sont limités par l'annotation du génome de référence, plus d'un tiers de ses gènes ne présentant pas d'homologues dans d'autres protéomes²⁷. Particulièrement, en ce qui concerne les lncRNA, bien que quelques bases de données existent, elles demeurent encore lacunaires et se focalisent majoritairement sur le génome humain. Une analyse prenant en compte l'ensemble des transcrits par lignée, comme une étude de réseau de coexpression (potentiellement en utilisant la méthode WGCNA⁴⁹), pourrait offrir une opportunité d'approfondir les réponses spécifiques aux différentes conditions d'exposition.

Par ailleurs, pour souligner davantage l'importance des ARN longs, il est à noter qu'un ARN long nommé DAPALR a été caractérisé chez l'espèce *Daphnia magna*⁵⁰. Celui-ci joue un rôle crucial dans la différenciation sexuelle des ovocytes en réprimant le régulateur Shep, lequel à son tour inhibe l'activation du gène doublesex1, contribuant ainsi à la formation des mâles et influant sur le cycle de

⁴⁴US EPA TG OPPTS 850.1500

⁴⁵OECD TG 240

⁴⁶US EPA TG OCSP 890.2100/740-C-15-003

⁴⁷OECD TG 233

⁴⁸Barata *et al.*, 2017, *Science of The Total Environment*, 579, 1073-83

⁴⁹Langfelder & Horvath, 2008, *BMC Bioinformatics*, 9, 1, 559

⁵⁰Kato *et al.*, 2018, *Current Biology*, 28, 11, 1811-1817

reproduction sexuée de ces organismes. En ce qui concerne la perturbation endocrinienne induite par la MIT chez le zebrafish²⁵, il a également été établi que des ARN non codants (en l'occurrence des micros ARN) jouent un rôle dans la régulation des hormones thyroïdiennes.

Dans cette optique, l'exploration d'une régulation épigénétique apparaît comme une piste digne d'intérêt. En somme, les effets d'une exposition chronique multigénérationnelle à la MIT ont mis en évidence une variabilité interclonale et intergénérationnelle, ainsi qu'un potentiel effet endocrinien, et ceci à de faibles concentrations. Ces conclusions indiquent que cette molécule pourrait avoir des conséquences significatives sur les écosystèmes, en particulier les milieux d'eau douce, engendrant non seulement des effets ponctuels, mais également des répercussions à long terme. En définitive, ces travaux soulignent l'importance d'adopter une approche évolutive en écotoxicologie⁵¹, particulièrement au vu du contexte de crise de la biodiversité.

⁵¹Straub *et al.*, 2020, *Nature Ecology & Evolution*, 4, 7,895-895

GENERAL INTRODUCTION - Freshwater pollution: investigating the ecological and evolutionary consequences of xenobiotic exposure through an evolutionary ecotoxicology framework

XENOBIOTIC IN FRESHWATER ENVIRONMENTS

Xenobiotics are chemical substances foreign to organisms, i.e., found within an organism while not naturally produced or expected to be present within this organism, either due to their sheer presence or because of their concentration. They include pesticides, polychlorinated biphenyls (PCBs), phthalates, volatile organic compounds, mycotoxins, heavy metals, and chlorines among the most common ones. These compounds can have natural or anthropogenic causes, and they can pose a significant threat to ecosystem integrity by polluting the environment, affecting human and environmental health. Freshwater biodiversity in particular is facing a crisis due to water pollution, habitat degradation, overexploitation, invasion by exotic species, and flow modification (Dudgeon et al., 2006; Harrison et al., 2018). Therefore, there is a need to better understand the manifold mechanisms by which pollutants affect freshwater biodiversity and how populations adapt to them. The present thesis aims to explore the impact of an emerging pollutant on a keystone freshwater species by taking an evolutionary outlook on ecotoxicology.

Xenobiotics have been shown to exert a range of deleterious effects on organisms, including acute toxicity, changes in behaviour, alterations of reproduction, and developmental abnormalities. However, the consequences of these effects extend beyond individual organisms and can have significant impacts on entire ecosystems (Arnold et al., 2013). Moreover, these effects can have long-term impacts due to the persistence of xenobiotics in the environment (Rieger et al., 2002). Many xenobiotics are poorly degradable (or it takes a long time), and they may be released on a regular basis into the environment, leading to accumulation and potential exposure to organisms over extended periods (Sumpter, 2009). These long-term effects can result in the decline of sensitive species, disruption of food webs, and other ecological impacts, with consequences that can persist for years. Therefore, it is critical to understand the full extent of these effects and their implications for aquatic ecosystems health and resilience.

The presence of xenobiotic compounds from wastewater is an important issue that is of concern to organizations such as the European Environment Agency (EEA) and the United States Environmental Protection Agency (US EPA). These organizations aim to develop policies that protect the environment by identifying the most harmful substances, reporting their effects, and providing scientific guidelines on how to monitor and improve the quality of water bodies (Loos et al., 2013). In the European Union, several directives and regulations exist, from REACH, which governs the registration, evaluation, authorisation and restriction of chemicals, on the basis of *a priori* risk evaluation, to the Water Framework Directive, which provides a legal framework to assess and manage chemicals (Vermeulen et al., 2019). Methods for decontaminating freshwater inputs include treating wastewater and regularly improving treatment plants. However, technical and economic limitations are hindering the effectiveness of the xenobiotic removal process from wastewater (Štefanac et al., 2021). Compounds that disrupt endocrine function and pharmaceuticals and personal care products, which occur at trace levels and have unique chemical properties, pose a particular challenge for removal (Roccaro et al., 2013). The persistence of these compounds in the environment, combined with their release over time, increases the potential for long-term ecological impacts. Upstream of these procedures, the marketing of chemicals is regulated at the EU level, on the basis of *a priori* risk assessment (see REACH legislation).

To summarize, xenobiotics are compounds that can have natural or anthropogenic causes, and may pose a significant threat to ecosystem integrity by polluting the environment and affecting human and environmental health. These compounds can have deleterious effects on organisms, entire ecosystems, and persist for long periods. Global and local organizations are working to develop policies and processes to identify and manage harmful substances. However, removing xenobiotics from wastewater is challenging, particularly endocrine disruptors, pharmaceuticals and personal care products, and other trace contaminants. The persistence of these compounds in the environment increases the potential for long-term ecological impacts. This emphasizes the need to better understand the mechanisms by which pollutants affect freshwater biodiversity, which is the traditional focus of ecotoxicology, but also how populations might adapt to them.

TOWARDS EVOLUTIONARY ECOTOXICOLOGY

The need for an evolutionary perspective in ecotoxicology

The necessity to monitor environmental health hazards was introduced in the second half of the 20th century after the groundbreaking publication of Rachel Carson's "Silent Spring", who first alerted the world to the ecological consequences associated with organochlorine insecticides (Carson, 1963),

as well as a series of dramatic events induced by chemical contamination (Vasseur et al., 2021). One of these was the mercury poisoning caused by water pollution which notably affected the bay of Minamata (Jensen & Jernelöv, 1969), causing ecosystem hazards including human losses (Harada, 1995). This terrible event led to the discovery of the biomagnification process, which describes how contaminant tissue concentrations increase in higher trophic levels. Such examples highlight the need to understand the mechanisms of contaminant toxicity on multiple scales and through multiple approaches.

Toxicology from an ecological perspective (“ecotoxicology”) aims to study the harmful effects of pollutants on living organisms and their relations within and between communities and their environment. However, these relations are bound to change in time, and current environmental risk assessment (ERA) procedures do not address real spatial or temporal exposure (Topping et al., 2020). The bioassays recommended for ecotoxicity testing currently available include usually short-term exposure (acute) tests on a representative set of terrestrial and aquatic species, as published under standardized guidelines and other guidance documents (see OECD). In some specific and priority situations only standardized longer exposure assays are performed (but see 2-generation reproductive toxicity tests recently developed to improve endocrine disruption assessment; <https://www.oecd.org/env/ehs/testing/oecdworkrelatedtoendocrinedisrupters.htm>). ERA outlines are flawed and lack realism in many ways, such as not considering indirect effects or the effects of multiplying stressors, or relying on resilient ecosystems with large sources of connected pristine populations, which results in overestimation of recovery; as shown e.g., following pesticide application (Caquet et al., 2007; Brock et al., 2010; Topping et al., 2014). For example, based on an agent-based model, Topping et al. (2014) reported significant limitations of plot experiments to predict population reductions in landscapes where pesticides are used. Furthermore, some impacts take time to emerge so that any acute assessment would miss long-term impacts.

As a result, long-term ecotoxicological effects are largely underestimated. These effects include delayed, transgenerational, and evolutionary effects, as continuously documented in the literature (Coutellec & Barata, 2011, 2013; Brady et al., 2017). Especially in fluctuating environments, evolutionary effects should not be neglected, as many studies have shown that the time-rate scaling of phenotypic evolution can be quicker than expected under the standard model of population genetics (Hairston et al., 2005; Messer et al., 2016), and that this rate of phenotypic change is increased in human-altered environments (Hendry et al., 2007). Xenobiotics can also be responsible for rapid evolutionary change, such as the rapid evolution of genetic resistance to antibiotics (Munita & Arias, 2016) or pesticides in target as well as non-target species (Major et al., 2018; Hawkins et al., 2019).

Therefore, an evolutionary perspective needs to be included in ecotoxicological aspects of ERA (Brady et al., 2017; Straub et al., 2020).

Mechanisms and consequences of xenobiotic resistance

The molecular basis of xenobiotic resistance can be divided into several classes, including constitutive overproduction or underproduction of a gene product, disruption of pathways involving targets or receptors, and gene regulation induced by environmental signals (Coustau et al., 2000). In a population exposed to a new substance or pollutant, the evolution of genetic resistance can result from different processes, including selection of advantageous variants from standing variation, de novo point mutation, and horizontal transfer from another species (Hawkins et al., 2019).

Therefore, at appropriate concentrations, myriad contaminants can induce changes in populations via several mechanisms including (Figure i), reduction of population size, direct genetic changes (i.e. mutations) or changes in genetic patterns at the population-level via selection (Belfiore & Anderson, 2001). Population-level adaptation may be the result of differential survivorship or reproductive output, resulting in fitness differences among individuals. Whatever the cause, these adaptations rely on standing genetic variation in the population.

Adaptation to xenobiotics can have ecological and evolutionary repercussions on different scales. Genetic processes at the population level induced by contaminants can influence ecological interactions at the community level. Empirical support for this can be found in studies such as those showing that the selection of insecticide-resistant daphnid clones mitigated algal blooms (Bendis & Relyea, 2016; Loria et al., 2022). In turn, community-level interactions may affect within-species genetic processes in response to chemical stress, as suggested in natural gammarid populations exposed to pesticides, in which the evolution of genetic resistance seems impeded by high invertebrate species diversity and associated interspecific competition (Becker et al., 2020).

The evolution of resistance can also incur direct or indirect physiological costs that affect the fitness of organisms in different contexts (Zbinden et al., 2008; Siddique et al., 2020). Once the xenobiotic pressure is relaxed, the evolved resistance should then face counter-selection (Coustau et al., 2000).

To gain a better understanding of evolutionary ecotoxicology mechanisms, and to be able to extrapolate the effect of toxic substances from laboratory experiments, it is essential to evaluate effects on fitness instead of focusing strictly on survival (Straub et al., 2020). Likewise, accounting for variability between organisms (Roubeau Dumont et al., 2019), as well as intra-specific variation in response to xenobiotics, in addition to long-term (i.e. multigenerational) studies is essential in defining

the extent of a pollutant's potential impact. Finally, molecular studies, especially through “omics” approaches may yield insights into the genetic and physiological effects of contaminants on non-target species (Bickham et al., 2000; Belfiore & Anderson, 2001; Snape et al., 2004; Brady et al., 2017).

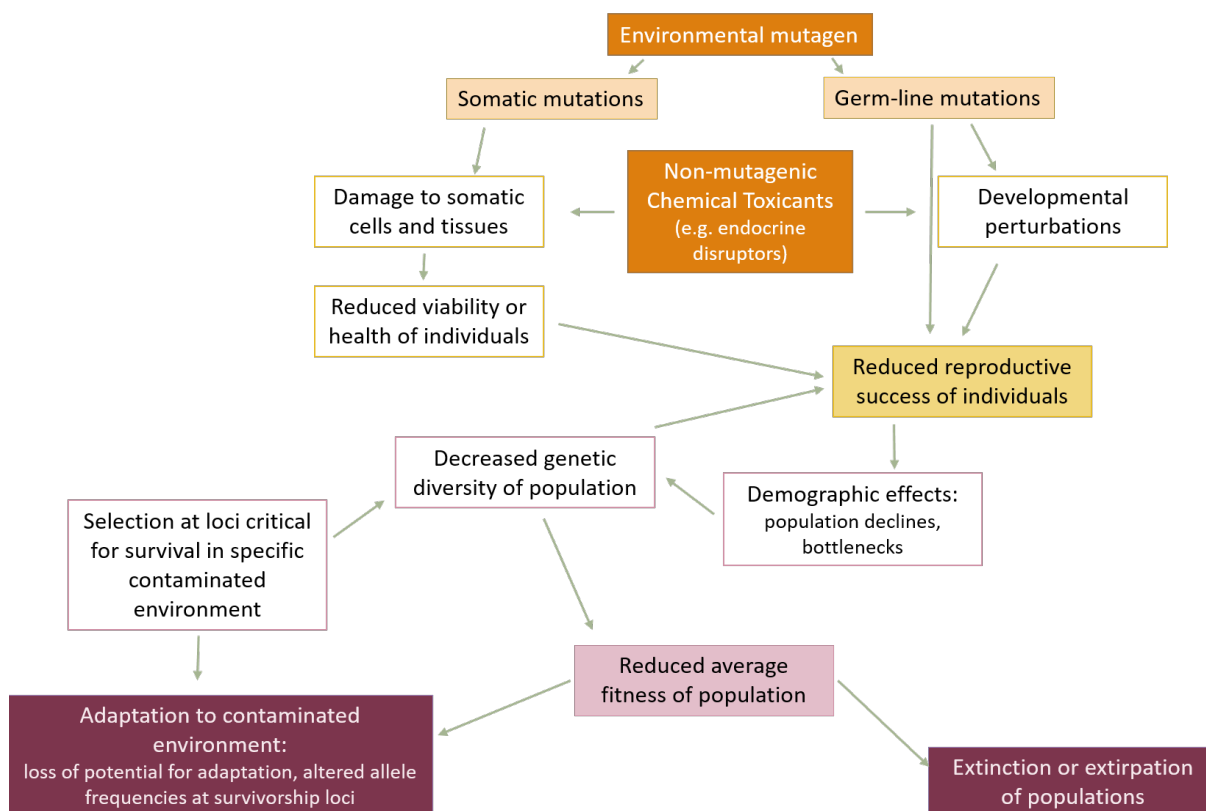


Figure i. Model to illustrate the inter-relationships among factors related to chemical contamination of the environment with decreased genetic diversity of populations (Bickham et al. 2000).

Omics approaches and case studies in evolutionary ecotoxicology

High-throughput methods, referred to as “omics”, have revolutionized the study of organisms and their responses to the environment, providing a more comprehensive view of biological traits at different levels of complexity. In a review comprising 648 studies published between 2000 and 2020, Ebner (2021) identified transcriptomics as the most frequently used method (43%), followed by proteomics (30%), metabolomics (13%), and multiomics (13%). Recently, there has been a trend towards combining omics technologies. The term “ecotoxicogenomics” was proposed by Snape et al. (2004) to describe the application of omics in an ecotoxicology framework, with the ultimate goal of deciphering the phenotypic and genotypic basis of fitness and its response to xenobiotic exposure using a molecular-to-population approach (Figure ii). Here, we explore some examples of omics methods and their applications in evolutionary ecotoxicology.

Transcriptomics is used to both quantify and identify RNA, and is frequently studied as a proxy for gene expression. In response to environmental changes, particularly in the context of xenobiotic exposure, it can be used to identify pathways involved in detoxification and stress responses. Gene expression signatures may be derived from expression patterns elicited by contaminants or groups of contaminants (Garcia-Reyero et al., 2009; Schirmer et al., 2010; Viñuela et al., 2010; Pfaff et al., 2021). For instance, Viñuela et al. (2010) demonstrated that exposure to two pesticides, chlorpyrifos and diazinon, induced different transcriptional responses in *Caenorhabditis elegans* depending on whether the organism was exposed to a single or a mixture of chemicals, with some responses overlapping.

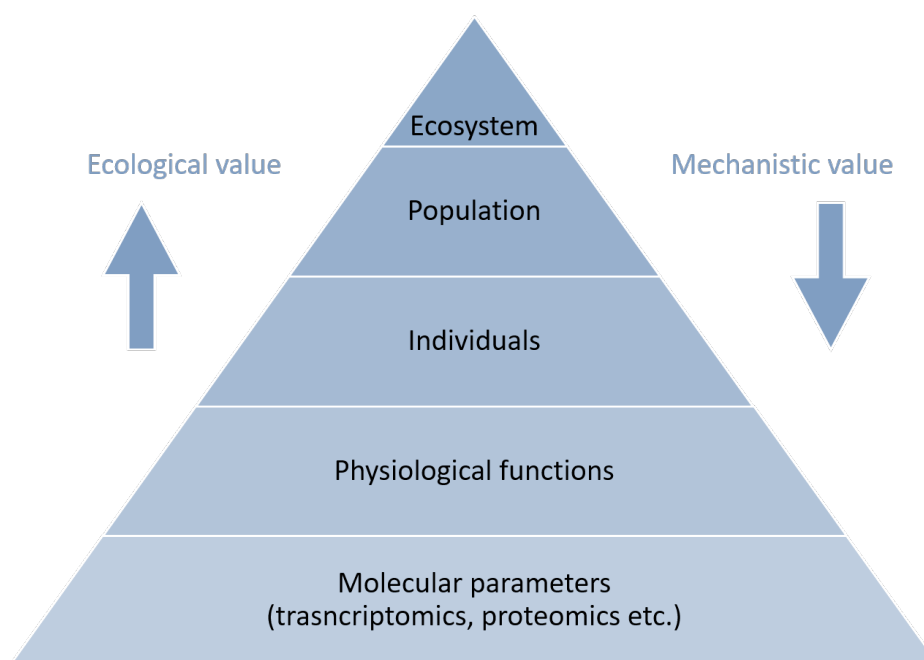


Figure ii. Conceptual framework for ecotoxicogenomics (Snape et al., 2004)

Proteomics focuses on characterizing protein function, modifications, interactions, and localization (Graves & Haystead, 2002). This intermediate scale between genes and phenotypes reflects the many mechanisms that interfere between transcription and the final state of a protein (Figure iii). In toxicology, proteomics allows the identification of biomarkers by studying changes in protein expression and modifications following toxic treatment (Apte & Mehendale, 2005). For example, Koenig et al. (2021) applied simultaneously proteomics co-expression networks to study responses to cadmium and two insecticides (pyriproxyfen and methoxyfenozide) in *Gammarus fossarum*. This method revealed specific functional groups of responses to each of the xenobiotics.

Metabolomics aims to profile changes in low molecular weight organic metabolite levels and pathways (Bundy et al., 2009), potentially providing information on the consequences of adaptation or adverse effects rather than regulation on the molecular scale (Shahid et al., 2021). Metabolomics approaches are ideal for the study of adaptive response to xenobiotics, such as the interaction

between natural stressors and chemical exposure. For instance, (Shahid et al., 2021) observed a significant synergetic effect of food depletion and insecticide stress at the metabolic scale in *D. magna* exposed to esfenvalerate, even at very low concentration of pesticide.

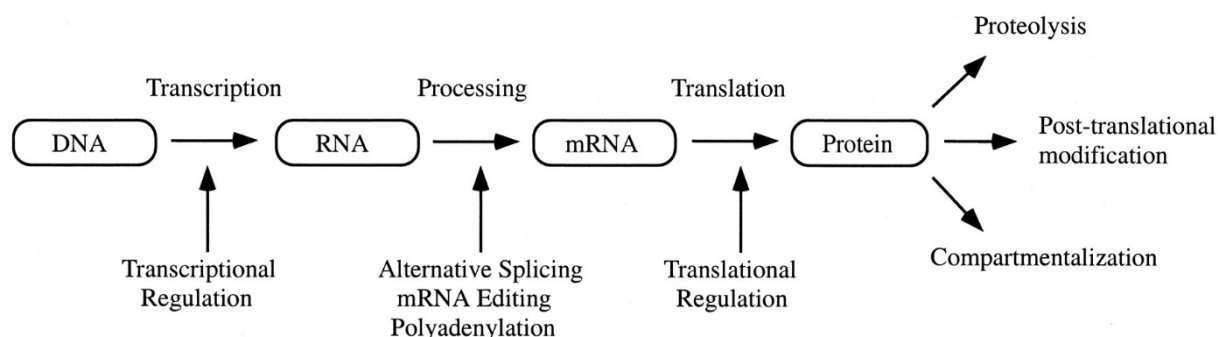


Figure iii. Mechanisms by which a single gene can give rise to multiple gene products. Multiple protein isoforms can be generated by RNA processing when RNA is alternatively spliced or edited to form mature mRNA. mRNA, in turn, can be regulated by stability and efficiency of translation. Proteins can be regulated by additional mechanisms, including posttranslational modification, proteolysis, or compartmentalization. (Graves and Haystead, 2002).

Challenges and limitations of omics approaches in evolutionary ecotoxicology

Omics approaches have the potential to provide valuable insights into the effects of environmental stressors on biological systems, providing insights into mechanistic/molecular linkages between traditional ecotoxicological endpoints and life-history characteristics. However, integrating large and often heterogeneous data can pose challenges and difficulties in interpreting results. One major challenge is translating measurements in model species exposed to model stressors into predictions of impacts for a broader range of species and ecosystems (Ebner, 2021). To address this issue, it is essential to develop predictive models that incorporate the impacts of multiple stressors across different levels of biological organization, including their interactions (Forbes & Galic, 2016). In addition, it has been suggested that emphasis should be put on trying to understand mechanisms using model species with complete genomes (Leung, 2018).

However, there are also challenges related to the reproducibility of data acquisition and data analysis, and quality control for individual and combined omics layers is necessary (Canzler et al., 2020). Furthermore, the rapid pace of omics technology development means that regulatory decisions may be obsolete by the time a course of action is decided.

Despite these limitations, omics information can benefit environmental risk assessment by providing more information on biological responses, and serving as an additional line of evidence (Leung, 2018). Omics approaches can be applied in environmental management domains, such as

identifying biomarkers and developing rapid detection tools for pollution monitoring, or establishing relationships between ecosystem function and chemical levels in the environment (Figure iv).

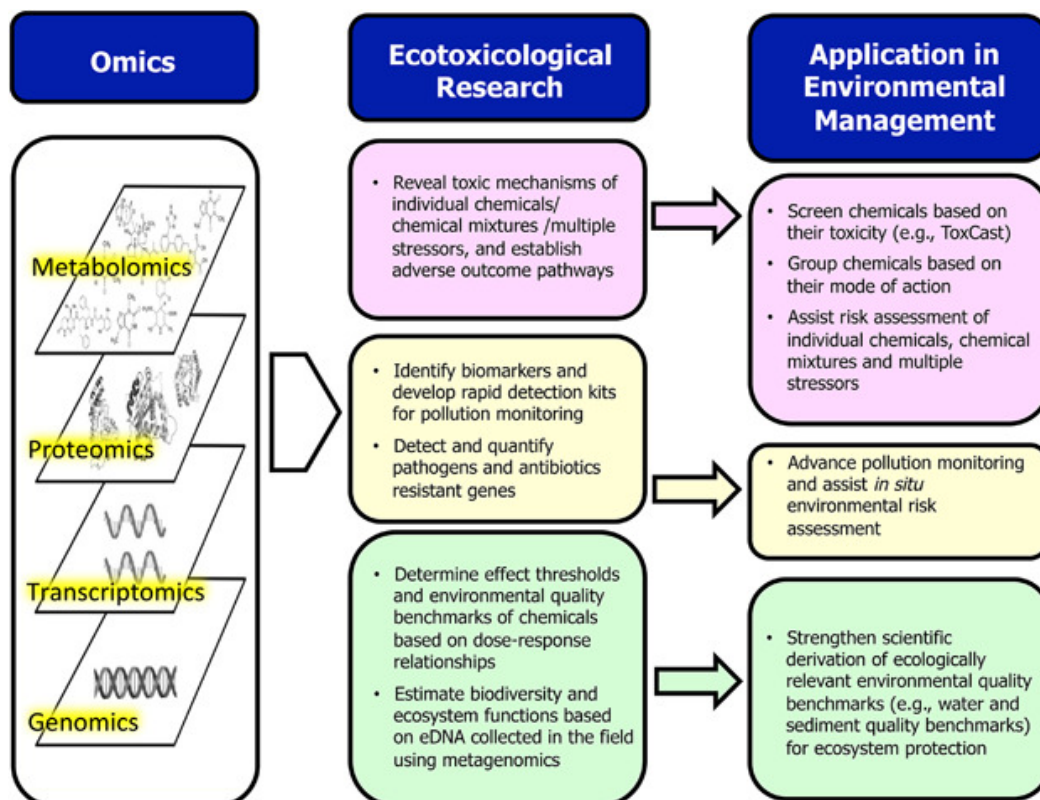


Figure iv. A schematic diagram to illustrate some examples of how omics and omics-generated information can be employed to assist environmental risk assessment and management of chemicals. eDNA = environmental DNA; ToxCast = Toxicity Forecatser (Leung, 2018)

We discussed the need for an evolutionary perspective in ecotoxicology to understand the mechanisms of contaminant toxicity on multiple scales and through multiple approaches. Current ERA schemes do not address real spatial or temporal exposure, and are flawed in many ways. Long-term ecotoxicological effects are largely underestimated, including delayed, transgenerational, and evolutionary effects. Xenobiotic resistance, its mechanisms and consequences, and how it can have ecological and evolutionary repercussions on different scales, are also discussed. We highlighted the importance of evaluating fitness, accounting for variability between organisms, and long-term and molecular studies, especially through omics approaches. Ultimately, integration across omics measures can lead to a deeper understanding of how organisms respond to stressors, and these measures can be used to develop models to predict phenotypes and outcomes (Ritchie et al., 2015).

DAPHNIA PULEX AS A MODEL SPECIES

A “model species” refers to a species that is used as a representative of a larger group of organisms. In ecotoxicology, they are chosen based on several factors including their sensitivity to xenobiotic stress and their ecological role (Figure v). In evolutionary biology, model species are used to investigate the genetic basis of complex traits, such as morphology, behaviour, and life history. Moreover, the purpose of model species is to be able to reliably compare, for example, the effects of different xenobiotics (Orsini, Gilbert, et al., 2016; Leung, 2018). Water fleas are one of these model systems that has been used for hundreds of years, as they possess a large panel of qualities which we will investigate here (Ebert, 2022).

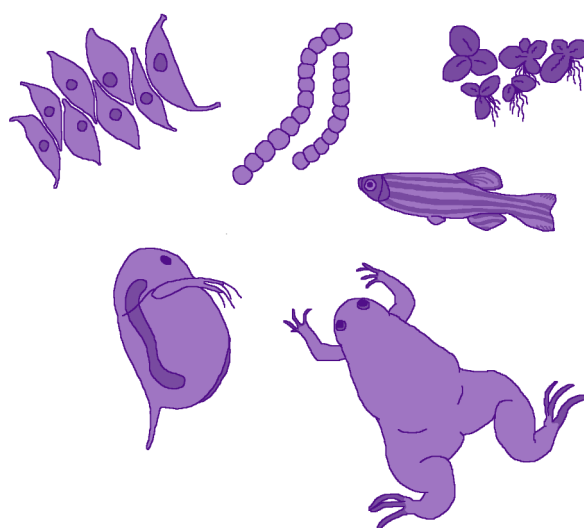


Figure v. Examples of aquatic model species for ecotoxicology ERA: *Raphidocelis subcapitata* (freshwater algae), *Skeletonema costatum* (saltwater algae), *Lemna* spp., *Danio rerio*, *Daphnia* spp., and *Xenopus laevis*.

Daphnia (Crustacea: Cladocera) are established model organisms in various fields of research, including ecology, ecotoxicology, population genetics, and genomics (Shaw et al., 2008; Colbourne et al., 2011; Orsini, Gilbert, et al., 2016; Brander et al., 2017). Their high sensitivity to environmental stressors, their abundance and widespread distribution in freshwater ecosystems, in addition to their short generation time and high reproductive output are compelling arguments for their study. One of their specificities is that *Daphnia* are cyclically parthenogenetic, alternating between sexual and asexual phases (Figures vi and vii), although obligatory asexual lineages exist too (Tucker et al., 2013). The asexual phase can be maintained in favourable conditions, thus enabling the maintenance of clonal lineages in laboratory. The use of clones and populations with genetic homogeneity allows for the control of genetic variation in experimental procedures. For instance, in established ecotoxicological risk assessment protocols such as the *Daphnia magna* reproduction tests n°202 and n°211 of the OECD (2004, 2012), single genotypes are used. Whereas the use of multiple genotypes

can specifically help improve the understanding of intraspecific variation on *Daphnia* response to stimuli (Tams et al., 2018; Chain et al., 2019; Ilić et al., 2021; Romero-Blanco & Alonso, 2022). Finally, with the increasing availability and affordability of genetic and genomic techniques, genome assemblies for eight different species of *Daphnia* are now available (Table i).

Table i. Partial list of *Daphnia* species genomes available on the National Center for Biotechnology Information (NCBI) library (www.ncbi.nlm.nih.gov/data-hub/genome/?taxon=6668, April 2023)

Organism Name	Assembly Accession	Assembly Name	Assembly BioProject Accession	WGS project accession
<i>Daphnia sinensis</i>	GCA_013167095.2	Dsi	PRJNA587065	WJBH02
<i>Daphnia dubia</i>	GCA_013387435.1	dubia_v0.01	PRJNA616071	JAAVJA01
<i>Daphnia obtusa</i>	GCA_016170125.1	FS6 v1.0	PRJNA598691	JAACYE01
<i>Daphnia magna</i>	GCA_020631705.2	ASM2063170v1.1	PRJNA738190	JAIFAF01
<i>Daphnia pulex</i>	GCA_021134715.1	ASM2113471v1	PRJNA777597	
<i>Daphnia pulicaria</i>	GCA_021234035.2	SC_F0-13Bv2	PRJNA762352	JAJAGA01
<i>Daphnia carinata</i>	GCA_022539665.3	CSIRO_AGI_Dcar_v0.2	PRJNA798159	JAKKWT02
<i>Daphnia galeata</i>	GCA_918697745.1	Dgal_M5_v1	PRJEB42807	CAKKLH01

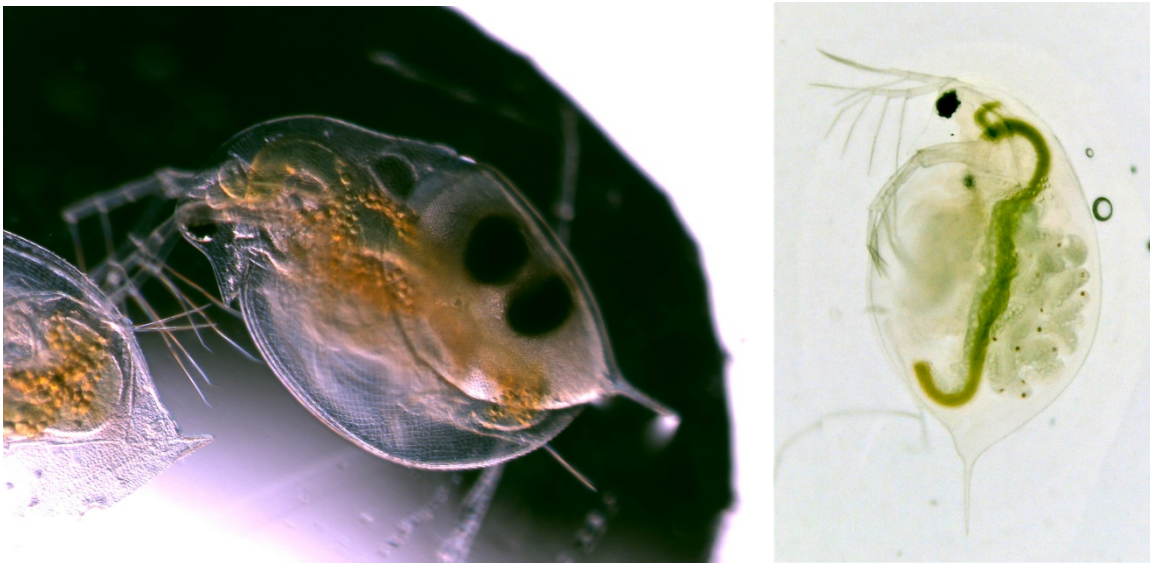


Figure vi. Mature *Daphnia pulex* females holding a fertilized egg (left) or parthenogenetic embryos (right) in their breeding pouch.

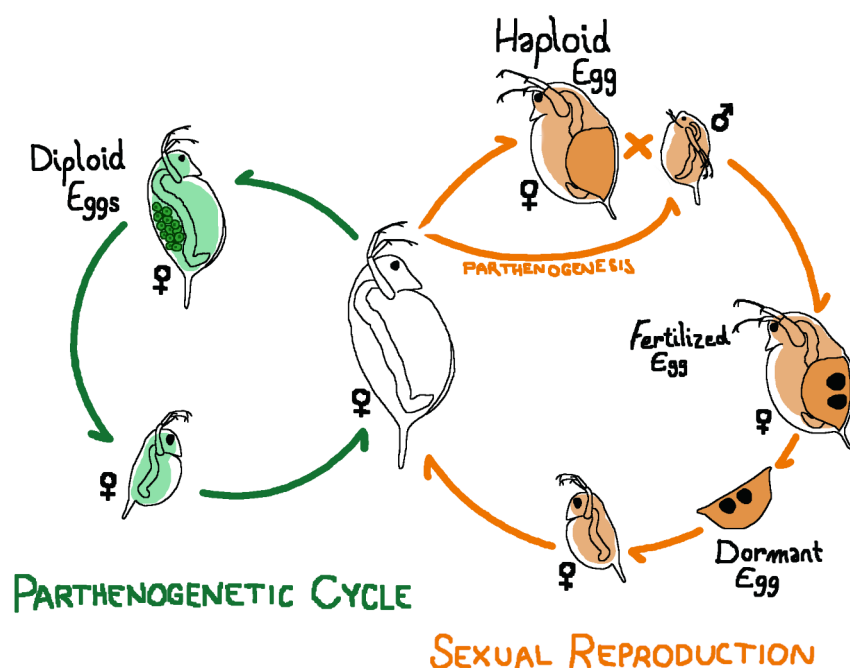


Figure vii. Daphnia life-cycle rotate between parthenogenetic cycles in stable conditions, and sexual reproduction when the situation deteriorates.

The unique attributes of daphnids make it a well-suited model system for ecology, evolutionary biology and toxicology, and bridge the different disciplines in which they are used (Shaw et al., 2008).

Daphnia have been extensively used to study genetic adaptation and evolution of stress response (Eads et al., 2008; Miner et al., 2012; Ebert, 2022), and to investigate gene expression and regulation in response to xenobiotics (Asselman et al., 2013; Suresh et al., 2020; Pfaff et al., 2021; J.-S. Lee et al., 2023). By taking advantage of available genomic tools and resources, daphnia are also integrated into multilevel-framework studies (H. J. Kim et al., 2015). Traditionally used as a toxicity test species (Shaw et al., 2008), daphnia are still ERA standards, with dedicated protocols testing xenobiotic effects, from acute toxicity to multigenerational testing for assessment of endocrine disruptors through short-term juvenile hormone activity screening (OECD, 2004; Abe et al., 2015; Barata et al., 2017).

METHYLISOTHIAZOLINONE AS AN EMERGEANT POLLUTANT

From a risk assessment perspective, xenobiotics considered as priority compounds are monitored in products and the environment. However, pollutants of emerging concern may sometimes appear after accidents or dramatic events. In South Korea, the use of the biocidal mixture chloromethyl- and methylisothiazolinone (CMIT/MIT) in humidifier disinfectant caused fatal lung injuries, exposing the underestimated toxicity of isothiazolinones (D.-U. Park et al., 2017; S. Kim et al., 2021). First

acknowledgment of the risks however happened only in 2011, 9 years after the first victims died (Choi & Jeon, 2019). Since this tragic public health case, the toxicity of CMIT/MIT has received more attention. However, isothiazolinones have a widespread use as stabilisers in many cosmetics and household products, including molecules for which the literature about their toxicity is scarce. In particular, the applications of MIT (Figure viii) alone increased, including wall paints, detergents, shampoos, lotions, and wet wipes, leading to increased exposure of both humans and ecosystems (Bollmann et al., 2017; Kresmann et al., 2018; Silva et al., 2020).

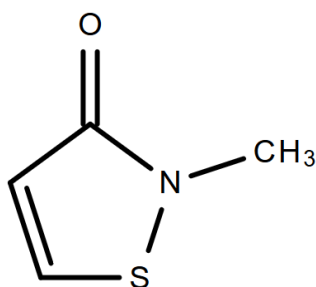


Figure viii. Methylisothiazolinone (2-méthylisothiazol-3(2H)-one, MIT, CAS no 2682-20-4)

In addition to the extension of environmental leaching, the removal of MIT from wastewater is very challenging, like other emerging pollutants are (Geissen et al., 2015; W.-L. Wang et al., 2019; X.-X. Wang et al., 2020). The increasing and prolonged exposure of freshwater non-target species to this molecule, coupled with the relative paucity of information available on its toxicity (Table ii), raise the necessity of an assessment of long-term MIT toxicity on aquatic species.

The mode of action of MIT lies on the production of free radicals and the destruction of thiol functions, which targets several proteins and enzymes involved in respiration and ATP synthesis, thus interrupting these processes and ultimately leading to cell damage and death in a non-selective way (Williams, 2007; Silva et al., 2020). Sub-lethal effects of MIT on non-target species include impaired wound healing, developmental damages, histopathological effects, and disruption of the thyroid endocrine system, with underlying molecular causes that involve genotoxicity and modification of genes expression regulation (Figure ix and Table ii) (Delos Santos et al., 2016; Capkin et al., 2017; S. Lee et al., 2022).

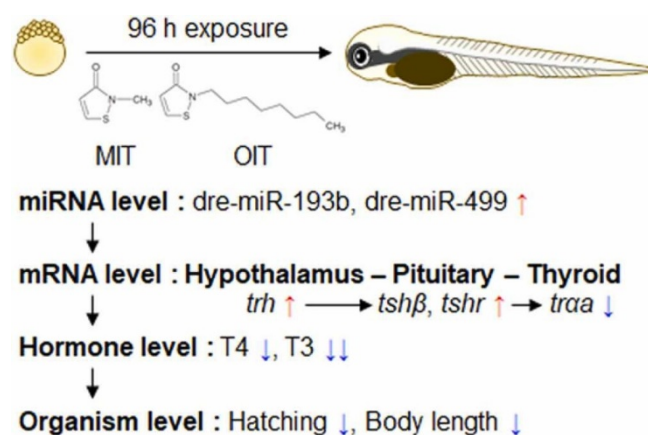


Figure ix. Effects of methylisothiazolinone and octylisothiazolinone on development and thyroid endocrine system in zebrafish larvae (Lee et al., 2022).

Table ii. Ecotoxicological data available for methylisothiazolinone

Species	Acute toxicity	Sub-lethal effects	References
<i>Dugesia japonica</i> (planarian)	LC50 = 2.06 (48h) - 4.83 (6h) mg/L	Delayed healing and impaired tissue regeneration	(Van Huizen et al., 2017; M.-H. Li, 2019a)
<i>Schmidtea mediterranea</i> (planarian)	LC50 = 5.75 mg/L (6h)		(Van Huizen et al., 2017)
<i>Girardia dorocephala</i> (planarian)	LC50 = 4.49 mg/L (6h)		(Van Huizen et al., 2017)
<i>Girardia tigrina</i> (planarian)	LC50 = 4.49 mg/L (6h)		(Van Huizen et al., 2017)
<i>Phagocata gracilis</i> (planarian)	LC50 = 8.06 mg/L (6h)		(Van Huizen et al., 2017)
<i>Xenopus laevis</i> (amphibian)		Delayed healing and impaired tissue regeneration	(Delos Santos et al., 2016)
<i>Oncorhynchus mykiss</i> (vertebrate)	LC50 = 5.51 mg/L (48h)	Histopathological effects, genotoxicity	(Capkin et al., 2017)
<i>Danio rerio</i> (vertebrate)		Developmental deficiencies, thyroid endocrine system disruption, NOEC = 30 µg/L	(S. Lee et al., 2022)
<i>Neocaridina denticulate</i> (arthropoda)	LC50 = 84.4 mg/L (48h)		(M.-H. Li, 2019a)
<i>Daphnia similis</i> (arthropoda)	LC50 = 0.8 mg/L (48h)		(M.-H. Li, 2019a)
<i>Daphnia magna</i> (arthropoda)	LC50 = 0.51-2.1 mg/L (48h)		(Kresmann et al., 2018; M.-H. Li, 2019a)

GENERAL OBJECTIVES

The overarching objective of this thesis is to determine the nature and extent of the effects that may arise in freshwater species following sustained exposure to emerging pollutants, with a specific focus on MIT.

To contribute to the environmental risk assessment of this biocide, we evaluated the ecotoxicity of MIT with a special attention to the potential effects on evolutionary processes. To this end, we conducted a series of experiments in controlled settings, using *Daphnia pulex* as a model species (Figure x). We started by evaluating the relative tolerance to MIT of various clonal lineages, thus incorporating intraspecific variability into a standard acute toxicity test (Chapter 1). Then we expanded the test to a multigenerational exposure, examining changes in response over time, while also accounting for intraspecific diversity. Chapter 2 relates the effects on fitness-linked traits, at the phenotypic scale, while Chapter 3 focuses on the gene expression scale. The questions we are trying to answer are: 1) Does MIT induces a stress even at low concentration? 2) Do daphnids respond similarly when they have been exposed for several generations? 3) Can MIT induce a response through indirect exposure? 4) Are there identifiable mechanisms of toxicity? 5) Do all clones have the same response to MIT exposure?

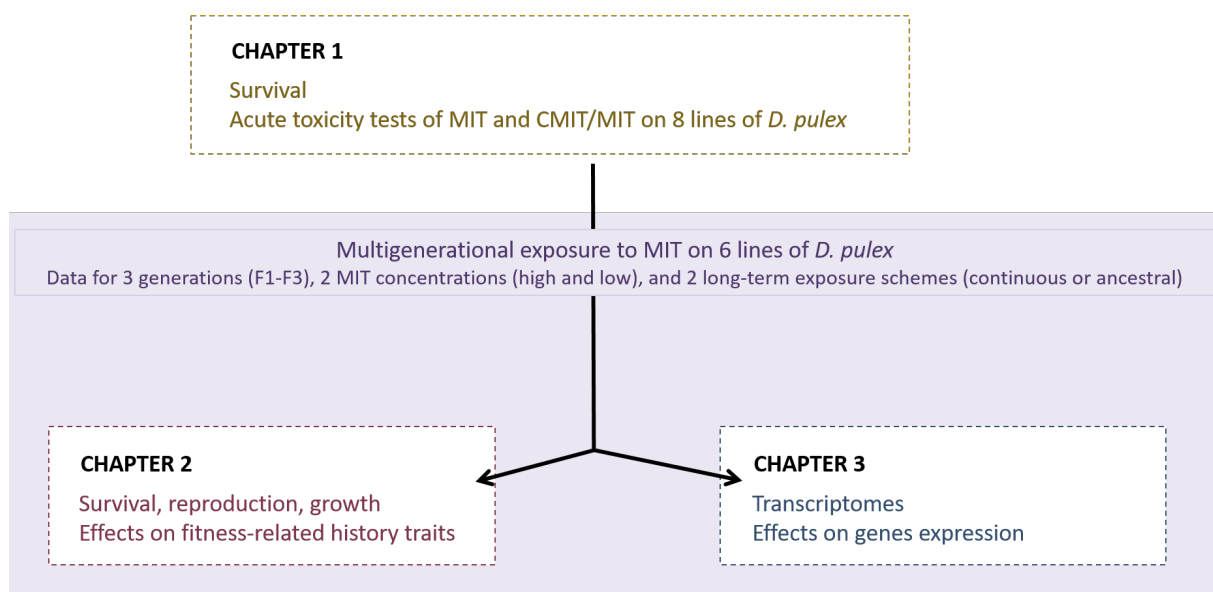


Figure x. Schematic overview of experiments that were carried out during the thesis

CHAPTER 1 - Variation of tolerance to Isothiazolinones among *Daphnia pulex* clones

ABSTRACT

Isothiazolinones are a family of broad-spectrum biocides widely used in industry and consumer products. Chloro- and methyl-isothiazolinones (CMIT and MIT) are documented as strong irritants, yet they are still used in a wide variety of applications, including cosmetics, cleansers, hygiene products and various industrial applications. The subsequent substantial release of these molecules from urban sources into freshwater environments, and their potential impacts on aquatic species, have nevertheless received little attention so far, with few studies reporting on the toxicity of either CMIT or MIT to non-target organisms. The present work addresses this current knowledge gap by evaluating CMIT/MIT (3:1) and MIT acute toxicity to *Daphnia pulex* (Cladocera), the two formulations most commonly used by manufacturers. Additionally, genetic diversity is known to be a major component of variability in phenotypic responses, although it is largely overlooked in typical toxicity tests. Subsequently the potential range of responses inherent to genetic diversity is rarely considered. Therefore, to account for intraspecific variation in sensitivity, the design involved eight clonal lines of *D. pulex* stemming from distinct natural populations or commercial strains. Clones exhibited strong variation in their responses, with lethal concentrations (LC50) ranging from 0.10 to 1.84 mg/L for the mixture CMIT/MIT, and from 0.68 to 2.84 mg/L for MIT alone. These intraspecific ranges of LC50 challenge the use of single clones of daphnids in standard ecotoxicological tests and the predictions based on their results. The present study brings new evidence that assessing ecological risk of chemicals while ignoring genotype diversity is neither ecologically relevant, nor a representative evaluation of the diversity of potential adverse outcomes.



Variation of Tolerance to Isothiazolinones Among *Daphnia pulex* Clones

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Abstract: Isothiazolinones are a family of broad-spectrum biocides widely used in industry and consumer products. Chloro- and methyl-isothiazolinones (CMIT and MIT) are documented as strong irritants, yet they are still used in a wide variety of applications, including cosmetics, cleansers, hygienic products, and various industrial applications. The subsequent substantial release of these molecules from urban sources into freshwater environments, and their potential impacts on aquatic species, have nevertheless received little attention so far, with few studies reporting on the toxicity of either CMIT or MIT to nontarget organisms. The present study addresses this current knowledge gap by evaluating the acute toxicity to *Daphnia pulex* (Cladocera) of CMIT/MIT (3:1) and MIT, the two formulations most commonly used by manufacturers. In addition, genetic diversity is known to be a major component of variability in phenotypic responses, although it is largely overlooked in typical toxicity tests. Thus the potential range of responses inherent to genetic diversity is rarely considered. Therefore, to account for intraspecific variations in sensitivity, our design involved eight clonal lines of *D. pulex* stemming from distinct natural populations or commercial strains. Clones exhibited strong variation in their responses, with median lethal concentration (LC50) values ranging from 0.10 to 1.84 mg/L for the mixture CMIT/MIT, and from 0.68 to 2.84 mg/L for MIT alone. These intraspecific ranges of LC50 values challenge the use of single clones of daphnids in standard ecotoxicological tests and the predictions based on their results. The present study brings new evidence that assessing ecological risk of chemicals while ignoring genotype diversity is neither ecologically relevant, nor a representative evaluation of the diversity of potential adverse outcomes. *Environ Toxicol Chem* 2023;42:805–814. © 2023 SETAC

Keywords: Aquatic invertebrates; Emerging pollutants; Evolutionary ecotoxicology; Freshwater toxicology; Risk extrapolation

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INTRODUCTION

Emerging pollutants are chemicals of various origins present in the environment that are not commonly monitored, and have not been studied in depth (Geissen et al., 2015). Their potential adverse effects on human health and ecosystems require action to be taken in favour of updated monitoring programmes and risk assessment tools (Geissen et al., 2015). Emerging contaminants include a wide variety of compounds such as pharmaceuticals, surfactants, pesticides, and disinfection by-products.

Isothiazolinones are broad-spectrum biocides used in many industries due to their efficacy against myriad microorganisms (Williams, 2007). Methylisothiazolinone (MIT, CAS n°2682-20-4) and chloro-methylisothiazolinone (CMIT, CAS n°26172-55-4) are two such molecules that can be found in cosmetics, household products, paint formulations, and industrial water treatment (Williams, 2007). CMIT and MIT are two of the most commonly found isothiazolinones on the market, and MIT was found to be the second most abundant biocidal active substance after citric acid in a review of 2963 products in 131 households of Northern Germany (Wieck et al., 2016). CMIT and MIT are two of the most commonly found isothiazolinones. They are sold notably in mixture in ratios of 3:1 (CMIT:MIT) as KathonTM (DuPont), and in several formulations of Acticide[®] (Thor GmbH). Until the 2000s, CMIT and MIT could not be synthesized separately, but as this process has been optimised, MIT – the less toxic of the two – is increasingly used on its own (Silva et al., 2020). Conversely, CMIT is too unstable to be used in isolation from MIT.

As for many biocides, isothiazolinone leaching from building materials or urban wastewaters first affects freshwater environments (Wittmer et al., 2011; Bollmann et al., 2017; Paijens, Bressy, et al., 2020). Bester et al. (2014) reported leaching of isothiazolinones in very high concentrations in run-off waters, up to 30 mg/L of MIT from acrylate renders. Indeed, since MIT has a poor affinity with microbial cells compared to other isothiazolinone molecules, it consequently requires higher doses for antimicrobial activity (Williams, 2007). In addition, due to its reduced toxicity, MIT is allowed and present in more numerous products than CMIT (ECHA, 2015a). Therefore, the benefit of using a less toxic molecule comes at the cost of a higher level of environmental contamination. Moreover, though CMIT is more toxic, it is also more quickly degraded than MIT, which is not readily biodegradable and thus more persistent in surface waters (Baranowska & Wojciechowska, 2013). Yet, like most other emerging pollutants, the presence of CMIT and MIT in the environment is not routinely monitored at present, regardless of the potential environmental hazards these molecules represent.

Isothiazolinones are remarkably efficient as biocides against microorganisms, but this efficacy also portends their potential as human and environmental hazards (Kresmann et al., 2018; Da-Silva-

Correa et al., 2022). Whilst sensitizing and allergenic effects of CMIT and MIT on human health have been well established, and have led to the restriction of their concentrations in personal care products (ECHA, 2015a; M. K. Kim et al., 2019), CMIT and MIT toxicity on aquatic organisms have received less attention (ECHA, 2014, 2015a). Known sublethal effects of MIT include delayed healing and impaired tissue regeneration in *Dugesia japonica* (planarian) and *Xenopus laevis* (amphibian), and in fishes, histopathological effects in *Oncorhynchus mykiss*, and developmental deficiencies in *Danio rerio* (Delos Santos et al., 2016; Capkin et al., 2017; Van Huizen et al., 2017; S. Lee et al., 2022). CMIT/MIT mixture has also been documented to induce brain damage, multiple morphological issues and decreased locomotion behaviour in *Danio rerio* (Cho & Kim, 2020; Chatterjee et al., 2021). To our knowledge, published results on CMIT/MIT acute toxicity to aquatic organisms are limited to those of Hu et al. (2014), who reported a median lethal concentration (LC₅₀) of 0.41 mg/L [0.33-0.49] (at 25°C) after 48h of exposure in the grass carp, and of Chatterjee et al. (2021) with a 96h-LC₅₀ of 0.44 mg/L [0.37-0.50] in zebrafish embryos. MIT acute toxicity seems to vary widely across aquatic invertebrate species, e.g., from 0.8 mg/L [0.55-1.19] in *Daphnia similis*, 2.06 mg/L [1.85-2.28] in *Dugesia japonica*, and up to 84.48 mg/L [70.70-100.94] *Neocaridina denticulate* (shrimp) (Li, 2019). Likewise, within species variation in sensitivity to MIT was also found to be substantial in *Daphnia magna*, with reported 48h-LC₅₀ values ranging from 0.51 mg/L [0.46-0.57] (Kresmann et al., 2018) to 2.1 mg/L (Li et al., 2016). Moreover, LC₅₀ values estimated in 5 planarian species after 6 hours of exposure ranged from 4.49 to 8.06 mg/L, supporting the idea that MIT lethality does not occur early upon exposure, at least in planarians (Van Huizen et al., 2017). Data on MIT toxicity to non-animal taxa are also scarce. In the microalgae *Scenedesmus* sp., MIT EC₅₀ (effective concentration) was 1.0 mg/L for growth inhibition (X.-X. Wang et al., 2018). Non-target prokaryotes may also be impaired. In wastewater-treatment settings, Amat et al. (2015) and Zeng et al. (2020) both reported a negative impact of MIT shocks on nitrification activity, with a modified composition of bacterial community in activated sludge. Given the extensive range of potential effects and affected organisms, and the scarcity of available data, it is crucial to expand our knowledge about environmental concentrations of isothiazolinones and their concomitant toxicity to freshwater ecosystems (Kresmann et al., 2018).

In addition to this lack of knowledge, it should be noted that ecotoxicity testing based on standard guidelines still suffers from a lack of ecological relevance, for several reasons. First, standardized bioassays tend to oversimplify environmental conditions and make results difficult to extrapolate to higher levels of biological organization (Crane et al., 2007; Forbes et al. 2008). Second, as genetic variability is deliberately disregarded in standard testing, evolutionary processes induced by toxicants cannot be addressed using current procedures of ecological risk assessment, heedless of increasing documentation and awareness of such potentials impacts (e.g., Medina et al., 2007; Coutellec & Barata, 2013; Weston et al., 2013; Oziolor et al., 2016; Brady et al., 2017). Genetic

variation is indeed predicted to correlate positively with population adaptive potential (see Willi et al., 2006). In accordance with this, Loria et al. (2022) showed that more genetically diverse populations of daphnids persisted longer under copper stress. Also, as reported above in *D. magna* exposed to MIT (Li et al., 2016; Kresmann et al., 2018), the occurrence of variation in sensitivity between clones or genotypes points to the risk of biased assessment when based on single-clone testing of species sensitivity and derived standard parameters (e.g., species sensitivity distribution; Posthuma et al., 2001).

Among freshwater organisms, daphnids are models of longstanding use in ecotoxicity testing (see e.g., OECD guidelines 202 and 211), notably due to their easy culture and short generation time, as well as their ecological status as keystone species in freshwater ecosystems. Here we assessed the toxicity of isothiazolinones to *Daphnia pulex* while emphasising within-species variation in sensitivity to highlight the importance of genetic diversity. To this end, the acute toxicity of MIT and of the mixture CMIT/MIT (ratio 3:1) to *D. pulex* was compared across eight clonal lineages stemming from various genetic and eco-evolutionary backgrounds. We hypothesized the lineages' 48h-LC₅₀ for MIT to be ranging between 0.5 and 2 mg/L, as previously found in *D. magna* and *D. similis* (see above). We expected that CMIT/MIT would induce higher mortality than MIT alone at similar doses and that clones showing higher tolerance to MIT would also be more tolerant to the mixture.

MATERIAL AND METHODS

Study clones and pre-experimental conditions

D. pulex clonal lineages established in the laboratory originated from several populations located in Brittany, France, as well as a laboratory culture provided by Aqualiment© (Supplementary Table S1-1). From each source, a single wild-caught individual was isolated and allowed to reproduce parthenogenetically to establish a clonal lineage. Lines were propagated for one year before biocide exposure. The clones were kept in dechlorinated tap water under standardized conditions (18°C, 16:8 L:D photoperiod) and fed with a mixture of two freshwater microalgae, *Chlorella vulgaris* and *Desmodesmus subspicatus*. Eight lines were selected (Supplementary Table S1-1) based on their potential differential response to biocide exposure (as estimated from preliminary tests), and their stability under lab conditions.

Experimental design and biocide exposure

MIT (95%; CAS 2682-20-4) and CMIT/MIT (secondary standard at 1.5% purity with a 3:1 ratio; CAS 55965-84-9) were purchased from Sigma-Aldrich. Pilot (i.e. range-finding) experiments were

conducted on batches of five-to-ten neonates of each line to assess the targeted range of toxicant concentrations. This range would include at least one low dose to ensure survival, and one high dose to induce death in all lines, with a minimum of three intermediate doses, in order to interpolate reasonable LC₅₀ values. Following pilot experiments, the next two series of nominal concentrations were prepared by dilution in filtered dechlorinated tap water, at the earliest two hours before the start of exposures: 0.25, 0.5, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.75, 3.0, 3.25, 3.75, and 5 mg/L for MIT and 0.06, 0.12, 0.15, 0.18, 0.24, 0.30, 0.36, 0.42, 0.5, and 0.6 mg/L for CMIT/MIT. Nominal concentrations were corrected by effective concentrations for statistical analyses.

At the start of the survival assessments, daphnia neonates (aged less than 24h) were isolated in a volume of 8 mL of control or treated water (n=50) in borosilicate glass test tubes (16 mm diameter). Renewal of the medium was performed after 24h of exposure, so as to mitigate biocide degradation and ensure a variation of less than 20% in concentration during the bioassay (see OECD (2004) and Table S3). Individuals were kept at the same temperature and photoperiod as culture conditions, but were not fed during the bioassay. Survival of each individual was examined after 24h and 48h of exposure, and tubes containing dead daphnids at 24h were emptied without renewal of the medium. Mortality was assessed using immobilization as a proxy (no observable movement for 20 seconds as recommended by OECD (2004)). The standard mortality criterion for test validity ($\leq 10\%$ of mortality in controls) was verified.

Analysis by ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)

UPLC-MS/MS has been successfully used for the determination of isothiazolinones in multiple matrices (Silva et al., 2020). In the present assay, the method was developed using an Ultra-Performance Liquid Chromatograph (Acquity, Waters) coupled with a quadrupole time-of-flight mass spectrometer (XEVO G2XS QTof, Waters). For the sake of sensitivity, repeatability, and reproducibility, the final measurements were performed on a triple-quadrupole mass spectrometer (XEVO TQD, Waters). For each treatment concentration, three random samples of contaminated water were collected at 0 and 24h, then pooled per time by concentration and filtered (GF/CA, 0.22 μ m, Phenomenex). An isotopically labelled internal standard (2-Methyl-d3-4-isothiazolin-3-one hydrochloride (MIT-D3), analytical standard, Sigma-Aldrich; CAS 1329509-49-0) corresponding to both target compounds was added before injection. Each sample was injected ten times.

Separation was performed with an Acquity UPLC BEH C18 column (50 x 2.1 mm inner diameter, 1.7 μ m particle size) from Waters, using a flow rate set at 0.7 mL.min⁻¹, column temperature of 30°C and injection volume of 5 μ L. The mobile phase through gradient elution was prepared by 0.1%

formic acid in water (A) and 0.1% formic acid in acetonitrile (B), using UPLC grade solvents. The mobile phase was initially started at 5% B at 0 min and increased to 15% B within 2.9 min, and then held at 5% until 3.5 min. Electrospray ionization was performed in positive mode with a capillary voltage of 3kV and a cone voltage of 30V. The acquisition was achieved in multiple reaction monitoring (MRM) mode, with a time scan of 0.25s. Collision energy and obtained values of the precursor and product ions are presented in Supplementary Table S2.

Mass spectral data acquisition and integration were respectively conducted with MassLynx® and TargetLynx® softwares (v.4.2, Waters). Measurement of effective concentration is described further in supplementary material (Supplementary Table S3), with calibration curves built from six concentrations including blanks. For samples with both molecules, CMIT/MIT concentration was quantified by adding CMIT and MIT individual values.

Survival analysis

All analyses were conducted in R version 4.0.0 (R Core Team, 2020). Average toxicity of CMIT/MIT and MIT was evaluated by modelling survival as a function of contaminant concentration, incorporating random variation amongst clonal lines and replicate tests. We fit generalized linear mixed models (GLMM) implemented in the 'MCMCglmm' package (Hadfield, 2010), using a logit link function and a binomial error distribution, with Markov chain Monte Carlo estimation. We compared a series of nested models, beginning with a maximal model including covariance between the intercept and coefficient terms of the logit model for both random effects, and proceeding with progressively less complex structures for each of the random terms. Ultimate model selection was based on minimizing the deviance information criterion (DIC). Models were run with a burn-in of 500 000 iterations, followed by 100 000 iterations from which each 100th point was sampled from the Markov Chain.

To test for differences between clonal lineages, additional GLMMs were run with both lineage and contaminant concentration allocated as fixed effects, and with random variation amongst replicate tests. We began with a model including both fixed effects and their interaction, followed by a model excluding the interaction term, and finally one including only contaminant concentration; note that all models shared the same random effects structure. These models were run with a burn-in of 200 000 iterations, followed by 100 000 iterations from which 1000 points were sampled from the Markov Chain. The significance of fixed effects terms was assessed via DIC comparison of nested models; significance of contrast coefficients of the parsimony model were profiled from the sampling chain.

Additionally, lethal concentrations (LC_{50}) for each clonal lineage were estimated with two methods implemented under the 'morse' R package (Baudrot & Charles, 2021). First, the mean survival rate at a given target time (48h) was described as a three-parameter log-logistic function of biocide concentration (supplementary material). Then, the same data were used to fit a toxicokinetic-toxicodynamic (TKTD) model using the GUTS framework (General Unified Threshold Model of Survival), under the assumption of differential sensitivity to chemical stress among individuals, i.e., the REDuced Individual Tolerance (RED-IT) version (Jager et al., 2011). GUTS modelling has the advantage of describing toxicant effects over time, leading to better fitting than the classical dose-response model, and can be used to predict the effect of the contaminant on survival for untested scenarios (such as time variable pulse exposure). The GUTS-RED-IT is a simple mechanistic model that describes the number of survivors in relation to time and external contaminant concentration. The internal concentration is assumed to be driven by the external concentration, via the "dominant toxicokinetic rate constant" (k_d , Supplementary Table S4), a parameter which portrays the speed at which the internal and external concentrations equilibrate (slowest compensating process, between either toxicokinetic elimination or toxicodynamic damage repair, which governs the overall dynamics of the scaled internal concentration). In turn, survival probability is lowered by background mortality (h_b parameter) and the internal concentration dependent threshold effect. The latter threshold effect distribution is described by the two parameters m_w (median) and θ (shape). In this model, once the threshold for one organism is exceeded, the organism dies immediately. Further descriptions and equations can be found in the original publication (Jager et al., 2011). For each clone, parameters were estimated using Bayesian inference, and posterior predictive checks were conducted to validate each model. To assess interclonal variation in sensitivity, we compared LC_{50} values and model parameters estimated from separated fits of the models.

RESULTS

The developed UPLC-MS/MS method successfully enabled the measurement of CMIT and MIT within the test range. High correlation coefficients (0.998-0.999, Figure S2 A and C) confirmed the linearity of the calibration curves in the range of tested concentrations. The quantification analysis showed concordance between effective and nominal concentrations (Figure S2 B and D), with a degradation of less than 10% over 24h for both CMIT/MIT mixture and MIT alone in all samples (Supplementary Table S3). Therefore, with the medium renewal at 24h, experimental concentrations met the 20% variation range prescribed as maximum by the OECD guideline n°202.

CMIT/MIT comparison

Parsimony dose-response models for both MIT and CMIT/MIT converged upon the same variance structure, incorporating random variation amongst lines in both the intercept (β_0) and slope terms (β_1), as well as random variation amongst technical replicates in the intercept (Table 1-1). A general view of the tolerance of daphnids to isothiazolinones (Figure 1-1) shows that the survival curve for CMIT/MIT is steeper than that of MIT, with an effect coefficient of CMIT/MIT concentration (-40.36) approximately 8-fold higher than that of MIT (-5.22; Table 1-1). This is consistent with the comparison of GUTS' kD parameter whose values among treatments (Figure 1-2) showed a globally quicker infiltration of the mixture ($mean = 1.37 \text{ day}^{-1}$, $sd = 0.281$) than of MIT alone ($mean = 0.997 \text{ day}^{-1}$, $sd = 0.351$). However, the difference between treatments (mixture vs MIT alone) was not significant in any lineage, as indicated by credible intervals.

Table 1- 1 Fixed-effects coefficients and variance estimates of random-effects for parsimony models of mean dose-survival curves. Interval estimates are profiled from the posterior density estimates sampled from 1000 points of the Markov chain.

	Est.	PDI _{0.025}	PDI _{0.975}	pMCMC
CMIT/MIT				
Model coefficient				
β_0	5.827	5.328	6.403	< 0.001
β_1	-40.365	-50.613	-27.480	< 0.001
Var. Comp.	Var	Var _{0.025}	Var _{0.975}	
Var{ β_0 Line}	0.2169	0.0003	0.7636	
		68.620		
Var{ β_1 Line}	278.4327	0	667.4698	
Var{ β_0 Rep Line}	1.3130	0.8085	1.7420	
Var{residual}	0.0488	0.0007	0.1581	
MIT				
Model coefficient				
β_0	5.152	4.596	5.746	< 0.001
β_1	-5.216	-7.078	-3.333	< 0.001
Var. Comp.	Var	Var _{0.025}	Var _{0.975}	
Var{ β_0 Line}	0.1308	0.0003	0.5308	
Var{ β_1 Line}	6.7456	1.2543	16.2181	
Var{ β_0 Rep Line}	2.2950	1.6260	3.1530	
Var{residual}	0.4548	0.0440	1.2830	

D.pulex showed a very large variance in global tolerance to increasing concentrations of both contaminants, as represented by credible intervals of the curves displayed in Figure 1-1. Indeed random variation amongst lines in the slope parameter of the logit models represented the greatest

fraction of total variance captured in each model (Table 1-1). Differences among lines are also supported by comparing dose-response models treating lineage as a fixed-effect: for both CMIT/MIT and MIT, model selection via DIC indicated significant line-by-concentration interaction effects (Table 1-2). This is further reflected in the significant differences in slope coefficients observed between many lines (Supplementary Table S6).

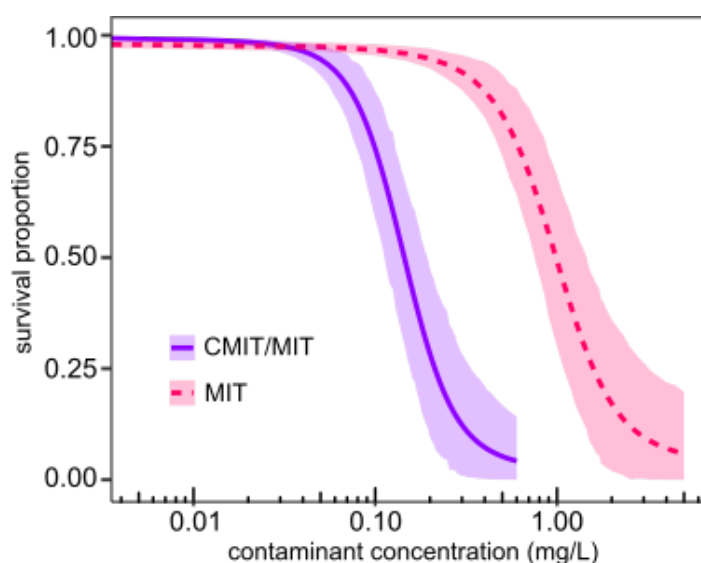


Figure 1- 1 *Daphnia pulex* sensitivity represented by its mean survival proportion (mean and credible intervals) after 48h of exposure depending on contaminant concentration (mg/L, log scale), estimated from GLMM models with lineage incorporated as a random term. Daphnids' sensitivity to the mixture (solid line) is about an order of magnitude higher than sensitivity to MIT alone (dashed line).

Table 1- 2 Comparison of nested hierarchical dose-response models. Random-effects structures are constant between models, and include only random variation amongst technical replicates in the intercept term. Models exhibiting the lowest values of the Deviance Information Criterion (DIC), are determined to represent a parsimony model, explaining the greatest amount of variance relative to model complexity.

Model Formulation	DIC
CMIT/MIT	
Conc + Line + Conc \times Line	1,540.73
Conc + Line	1,639.52
Conc	1,602.75
MIT	
Conc + Line + Conc \times Line	2,260.16
Conc + Line	2,314.13
Conc	2,288.54

The estimated values of 48h-LC₅₀ using GLMM (Figures 1-3B and 1-4B), GUTS-IT, or time-target analysis are presented in Table 1-3. The three methods reported consistent results, and the GUTS-IT model showed that CMIT/MIT mixture was almost one order of magnitude more toxic than MIT alone (LC₅₀ values: 0.10 - 0.37 mg/L vs. 0.68 - 2.84 mg/L, respectively). Clones displayed different levels of tolerance (Figures 1-3 and 1-4). In terms of tolerance curve shape, AL0 and SE5 differed widely from one another and from the rest, whereas the six remaining clones formed a more homogeneous group. Clones AL0 and SE5 were the most tolerant to MIT (LC₅₀ in mg/L: 2.84 and 1.84, respectively) and to CMIT/MIT (0.37 and 0.24, respectively), while at the other end, SE2 and LA0 were the most sensitive to MIT (LC₅₀ in mg/L: 0.74 and 0.68, respectively) and CMIT/MIT (0.10 and 0.13, respectively).

Table 1- 3 Lethal concentrations for 50% (LC50) at 48h values and 95% credible interval, in mg/L, computed with either GLMM, GUTS-RED-IT or an exposure-response log-logistic model for 8 clonal lines of *Daphnia pulex*.

Clone	CMIT + MIT			MIT		
	GLMM	GUTS-IT	Log-logit	GLMM	GUTS-IT	Log-logit
AL0	0.40 [0.34-0.47]	0.37 [0.35-0.39]	0.37 [0.36-0.39]	2.86 [2.50-3.19]	2.84 [2.66-3.03]	2.73 [2.59-2.88]
GO6	0.15 [0.13-0.17]	0.15 [0.14-0.16]	0.157 [0.149-0.164]	0.78 [0.64-0.91]	0.78 [0.70-0.87]	0.79 [0.70-0.92]
LA0	0.12 [0.10-0.14]	0.13 [0.12-0.14]	0.13 [0.12-0.14]	0.63 [0.47-0.80]	0.68 [0.60-0.75]	0.66 [0.59-0.89]
RE0	0.12 [0.11-0.14]	0.14 [0.13-0.15]	0.146 [0.137-0.155]	1.02 [0.88-1.15]	0.96 [0.83-1.08]	1.14 [0.96-1.24]
SE2	0.10 [0.08-0.12]	0.10 [0.09-0.11]	0.10 [0.09-0.11]	0.71 [0.56-0.87]	0.74 [0.66-0.82]	0.74 [0.65-0.86]
SE5	0.23 [0.20-0.26]	0.24 [0.22-0.25]	0.24 [0.22-0.25]	1.76 [1.53-2.00]	1.84 [1.74-1.95]	1.86 [1.74-1.98]
P16	0.13 [0.11-0.15]	0.13 [0.12-0.14]	0.13 [0.12-0.14]	0.96 [0.73-1.17]	1.08 [0.99-1.17]	1.12 [1.04-1.20]
PE7	0.13 [0.10-0.15]	0.13 [0.12-0.14]	0.14 [0.13-0.15]	1.00 [0.80-1.20]	0.92 [0.83-1.02]	0.96 [0.85-1.07]

DISCUSSION

Presence and risks to ecosystems

The present study provides data about CMIT/MIT and MIT acute toxicity to *D. pulex*, validated by the successful measurement of both compounds' concentrations. Regarding isothiazolinone detection and quantification in environmental waters, liquid chromatography methods coupled with mass spectrometry showed good performances (Speksnijder et al., 2010; Paijens, Frère, et al., 2020). In particular, to analyse CMIT and MIT in cosmetics, UPLC-MS/MS methods were developed with increased sensitivity, selectivity, and increased signal-to-noise ratio especially concerning MIT

detection (Wittenberg et al., 2015; Lee et al., 2020; Ducup de Saint Paul et al., 2021). Our results also support the use of UPLC-MS/MS as a fast and sensitive method for quantifying CMIT and MIT in water samples. Accurate and easy to handle analytical techniques are required for reliable and routine environmental monitoring as well as toxicity assessment, which remains challenging in the case of MIT (ANSES, 2016). Targeted screening studies were able to detect both CMIT and MIT in various natural aqueous and soil matrices, though with mixed success, in particular regarding MIT recovery rates (Speksnijder et al., 2010; Baranowska & Wojciechowska, 2013; Nowak et al., 2020; Paijens, Frère, et al., 2020; Paijens et al., 2021). MIT was found in concentrations from 0.2 to 0.9 µg/L in wastewaters from the Parisian basin (France) and 0.162 µg/L in a stormwater runoff collected in Silkeborg (Denmark) (Bollmann et al., 2014; Paijens et al., 2021). CMIT concentration reached 0.16 µg/L in combined sewer overflows (Paijens et al., 2021). Based on multiple assays, including acute toxicity to *D. magna*, Kresmann et al. (2018) calculated a predicted no effect concentration (PNEC) of 0.5 µg/L for MIT, i.e., a value below some reported environmental concentrations. Although still too fragmentary, these data and predictions strongly support the need for closer attention to these emerging biocides, both in terms of environmental analysis and of ecotoxicity testing. In this respect, our analytical methodology proved to be efficient.

Considering the higher chemical reactivity of the chlorinated molecule (Collier, Ramsey, Waigh, et al., 1990), we unsurprisingly found the mixture CMIT/MIT to be about seven times more lethal than MIT alone. Whether this increase reflects additive or synergistic interaction between the two molecules cannot be established from the present study, as the toxicity of CMIT alone could not be tested. As discussed before (see introduction), the lower lethal activity of MIT among all isothiazolinones (Williams, 2007) comes with high admitted concentrations in commercial products in a very large panel of products (Silva et al., 2020), which translates into increased discharges to the environment. In the meantime, MIT sub-lethal effects remain largely unstudied, stressing once again the current underestimation of risks to ecosystems.

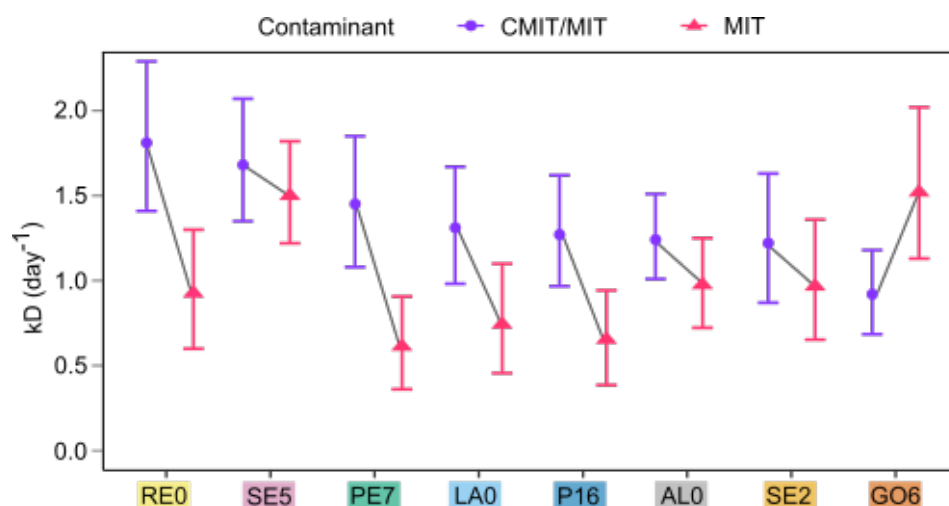


Figure 1- 2 Dominant rate parameter (kD , median and 95% credible interval) as estimated from separate fits of GUTS-IT models for each clonal lineage, contaminated with CMIT/MIT (points, purple lines) or MIT alone (triangles, pink lines). The kD parameter portrays the speed at which the internal and external concentrations equilibrate (see methods). estimated from GLMM models with lineage treated as a fixed effect and random variation amongst technical replicates.

Intraspecific variability

With respect to intraspecific variation, we observed that tolerance to either MIT or CMIT/MIT was highly dependent on the genotype assayed, with a LC_{50} value of the most sensitive clone about 4 times smaller than that of the least sensitive clone in both cases (Table 3). As indicative of genotype-by-environment interaction, these results suggest the possibility of selective processes induced by CMIT and MIT in exposed natural populations and to their subsequent genetic divergence, in particular from non-exposed ones. Evidences of microevolution due to chemical pollution in aquatic populations are increasingly documented (e.g., Oziolor et al., 2016; Major et al., 2018; Gouin et al., 2019). Recent studies revealed the development of insecticide resistance in populations of non-target species, such as the crustaceans *Hyallela azteca* (pyrethroids; Major et al., 2018) and *Gammarus* sp. (neonicotinoids; Shahid et al., 2018). In daphnids, Brans et al. (2021) found urban populations of *D. magna* to be more tolerant to chlorpyrifos than rural ones from the same geographical region (Flanders). In the same region, *D. magna* populations isolated from ponds located in agricultural areas presented specific pesticide resistance in line with local management practices (either chlorpyrifos or deltamethrin; Almeida et al., 2021). Romero-Blanco & Alonso (2022) also noted from database and literature review, that the sensitivity of aquatic species to contaminants depended strongly on the origin of populations, with either wild or laboratory-reared populations being the most tolerant, depending on the chemical. However, the persistence of the most tolerant populations may come not only with a reduction of genetic diversity but also with fitness or physiological costs (Coustau et al., 2000; Jansen et al., 2011). For instance, in *Gammarus pulex*, populations tolerant to the neonicotinoid clothianidin exhibited reduced fitness in pesticide-free laboratory conditions (Siddique et al., 2020).

In addition to a contribution to fill the knowledge gap on toxicity of CMIT/MIT and MIT to non-target organisms, this study provides clear evidence for a significant genetic component in *D. pulex* sensitivity to such a chemical stress. These findings point to the risk of current standard tests based on daphnids, which typically resort to single clone assays, inducing inaccuracy in environmental quality criteria (e.g. species sensitivity distribution; Posthuma et al., 2001) and eventually leading to over- as well as under-protective measures, depending on the sensitivity of the clone used. While the latter risk is of particular concern on ecological grounds, the former may also be important for manufacturers who would need to adapt to overly stringent measures, either by deeply modifying the production line or by putting more effort into finding other less toxic molecules of interest. Also, when experimentally assessing chemical toxicity at the community-level (higher-tiered approach), the choice of a monoclonal or a genetically diverse origin population is expected to influence the outcome (Loria et al., 2022). Furthermore, one might argue that interspecific variation overtakes intraspecific variability (Roubeau Dumont et al., 2019), but the relative importance of these seems to greatly depends on species and traits studied (Vanvelk et al., 2021).

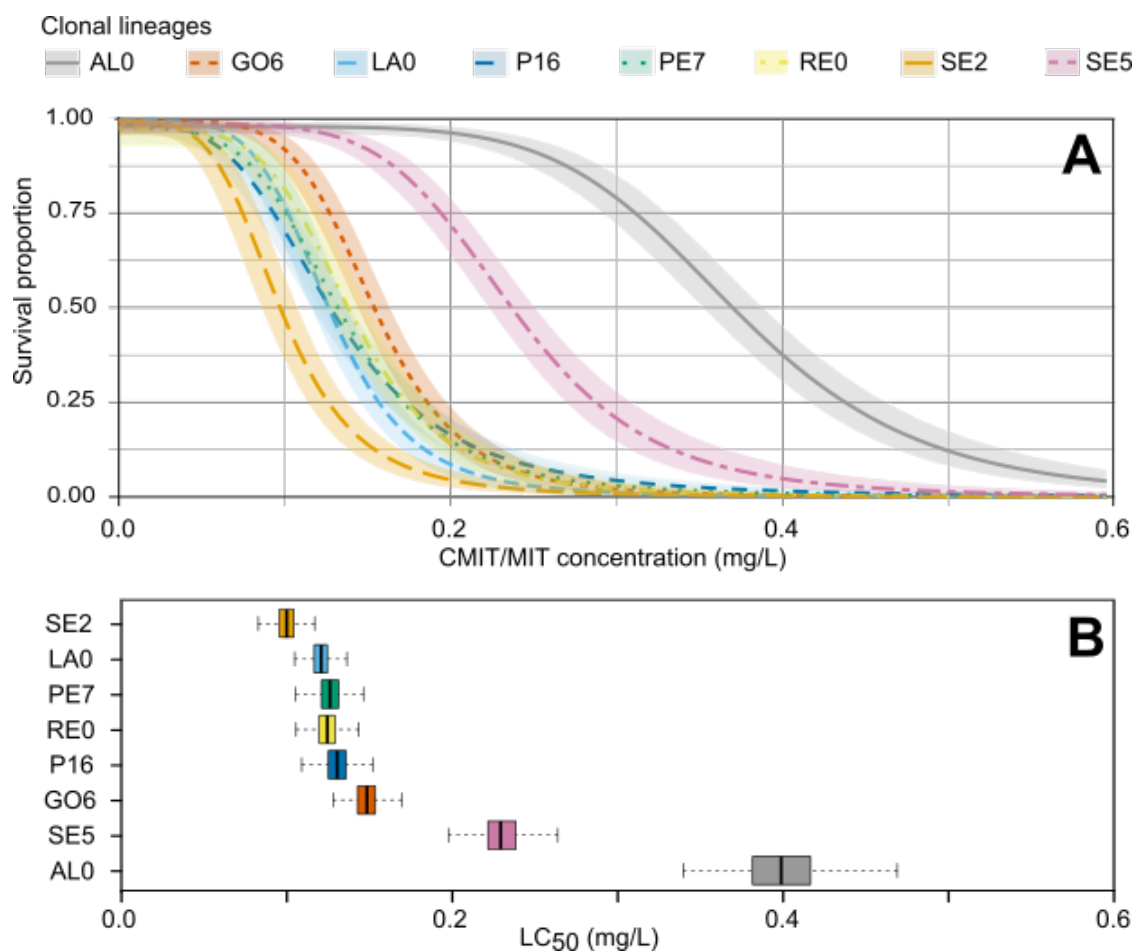


Figure 1- 3 Tolerance to CMIT/MIT after 48h of exposure of each *D. pulex* clonal population (n=50). In the upper panel (A), tolerance is expressed as the survival proportion, with the median survival and

95% credible interval estimated from separated fits of GUTS-RED-IT. In the lower panel (B), tolerance is expressed as 50% lethal concentration (median LC₅₀, quartile range, and 95% credible interval)

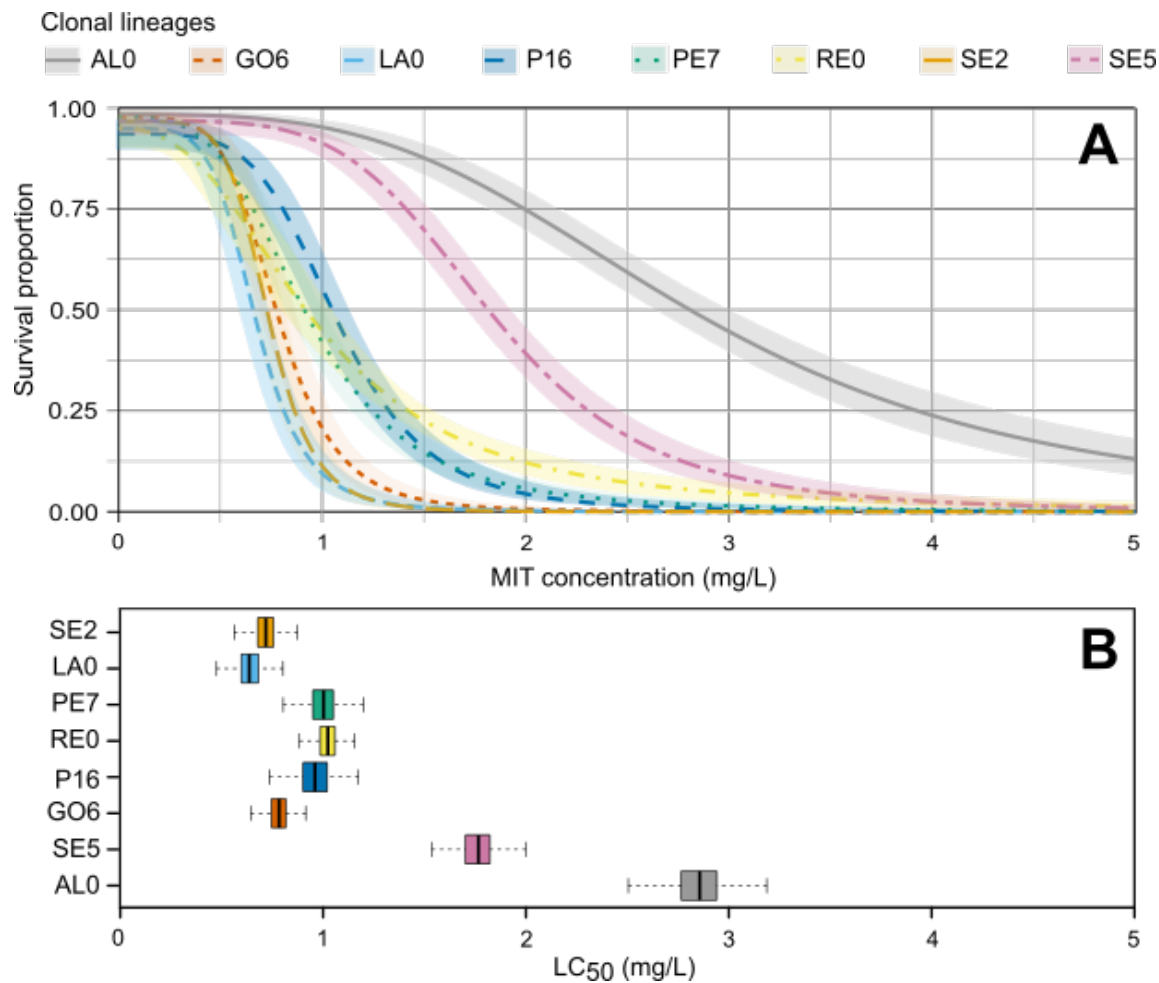


Figure 1- 4 Tolerance to MIT after 48h of exposure of each *D. pulex* clonal population (n=50). In the upper panel (A), tolerance is expressed as the survival proportion, with the median survival and 95% credible interval estimated from separated fits of GUTS-RED-IT. In the lower panel (B), tolerance is expressed as 50% lethal concentration (median LC₅₀, quartile range, and 95% credible interval) estimated from GLMM models with lineage treated as a fixed effect and random variation amongst technical replicates.

Using several lineages may help in refining the understanding of the mechanisms involved in toxicity and tolerance, particularly if a genetic basis for differential tolerance exists, as suggested by the amongst-line heterogeneity observed in the present study. The use of multiple genotypes is a cornerstone of eco-evolutionary experimental designs. For instance, in the case of daphnids, Orsini et al. (2016) recommend five to ten individuals genotyped at twenty neutral markers to obtain a good estimate of allelic richness of one population. More generally, the inclusion of evolutionary toxicology in ERA, for example through the study of the impact of reduced genetic diversity, has been discussed for decades (see Bickham et al., 2000), and leads have been opened up by focusing on more mechanistic and molecular approaches by using Adverse Outcome Pathways or high-throughput screening (Klerks et al., 2011; Côte et al., 2015; Oziolor et al., 2020). Besides documentation of

evolutionary impacts, the need for a unified understanding of such effects (see Brady et al., 2017) calls for harmonized methodologies and parameters that are meaningful for risk assessment. Yet, it is still not a common practice in ecotoxicological research, and the use of multiple genotypes is deliberately avoided in standard toxicity testing, despite regular warnings (Barata et al., 2002; Medina et al., 2007; Coutellec & Barata, 2011; Côte et al., 2015). The present study brings new evidence that ignoring genotype diversity is neither ecologically relevant nor sustainable, especially when considering that biodiversity in all its dimensions is now recognized as a protection goal in environmental risk assessment (EFSA Scientific Committee, 2016).

From acute toxicity to chronic exposure

Due to its extensive and wide uses, MIT is continuously discharged to the environment all year (and not seasonally like crop pesticides for example) through wastewater, and is degraded over a few days (Rafoth et al., 2007; Paijens, Bressy, et al., 2020). In the first chapter, water fleas were exposed to very high concentrations of biocides compared to that of environmental traces (ng/L scale, Paijens, Frère, et al., 2020). Low concentrations of contaminants are known to sometimes induce sublethal effects (e.g. on behaviour, on reproduction, or endocrine system disruption), with different underlying modes of action than acute toxicity. Yet extrapolating “safe” concentrations from daphnia immobilization test, such as determining no observed effects concentrations (NOEC) values directly correlated from LC50 (as 1:100, 1:1000, or 1:10000 fraction of LC50), is common, in contradiction with the known potential for low-dose risks (Liess & Ohe, 2005; Crane et al., 2007; Schäfer et al., 2012; Liess et al., 2019). In chapter 2, we therefore present results of a long-term multigenerational experiment conducted with relatively lower concentrations including a low dose that would be considered safe following MIT NOEC value for *D. magna* (NOEC 21d = 40 µg/L (DOW, 2015), maximum doses tested : 50 and 0.8 µg/L).

CHAPTER 2 - Multigenerational effects of mild isothiazolinone exposure on water flea fitness

ABSTRACT

In this chapter, we aimed to investigate the latent effects of mild concentrations of methylisothiazolinone (MIT, biocide) on the freshwater crustacean *Daphnia pulex* in various exposure setups. We found that both lethal and sub-lethal effects changed over generations and were evident in direct exposure scenarios, but not in parental exposure scenarios. Additionally, continuous exposure to environmental MIT doses caused significant adverse effects on this non-target species. We also highlighted the importance of considering intraspecific variability in toxicity assessments, as the intensity and temporality of the effects observed were dependent on the specific line of *D. pulex* studied. Overall, these findings underscore the potential long-term effects of xenobiotic exposure on non-target species and the need for careful consideration of intraspecific variability in toxicity assessments.

INTRODUCTION

Xenobiotics are exogenous molecules present in an organism in an abnormal way, either because of their origin or because of their high concentration. The introduction of xenobiotics into freshwater ecosystems can have a significant impact on natural populations, including mortality and sublethal effects such as changes in behaviour and physiology. On a longer term, they can also have consequences on evolutionary processes which, in turn, impacts ecological dynamics (Yamamichi, 2022).

From an evolutionary perspective, xenobiotics are consequential due to their potential effects on the genetic makeup of individuals via induced genetic mutations or DNA damage, as well as their potential to exert a selective pressure on exposed populations. For example, if a xenobiotic is toxic to certain individuals, those that are resistant or tolerant to it will be more likely to survive and reproduce. Over time, this can lead to local adaptations. Instances can be found of urban zooplankton populations being more resistant to some xenobiotics than their rural counterparts (Brans et al., 2021). Likewise, some populations located near agricultural fields were found to be more resistant than others collected in more isolated ponds (Bendis & Relyea, 2014).

The variability of responses by organisms depending on the context, and most particularly genetically based intraspecific variability, is the basis for selective evolution. For example, in the context of a freshwater ecosystems impacted by eutrophication and cyanobacterial blooms, Schwarzenberger et al. (2020) found a genetic basis (the positive selection of digestive protease) participating in local adaptation of *Daphnia magna* populations to cyanobacterial protease inhibitors. It must however be noted that resistance or tolerance can come with potential costs to fitness. For example, populations of parasite-adapted *D. magna*, or pesticide-tolerant *Gammarus pulex* populations had a lower general fitness compared to more susceptible populations (Zbinden et al., 2008; Siddique et al., 2020). Overall, xenobiotics can have a significant impact on the evolutionary processes of freshwater species, which needs to be better understood. Such assessments require exploring various mechanisms over multiple generations. This work aims to contribute to this by studying the effects of an emergent xenobiotic on a species of water flea.

The sensitivity of *Daphnia*, their short generation time, and cycles of clonal reproduction makes them ideal organisms for studying evolutionary dynamics, as illustrated above (Ebert, 2022). *Daphnia* have been used extensively as a model organism in ecology and ecotoxicology, with studies relating the effects of various types of contaminants over multiple generations, such as organic

contamination with fecal coliform (Chatterjee et al., 2019), inorganic contamination with silver nanoparticles (Ellis et al., 2020), or pollution with pesticides like chlorpyrifos (Maggio & Jenkins, 2021).

On the other hand, methylisothiazolinone (MIT) is a biocide widely used in particular as stabilisers in cosmetics and is an emerging pollutant (Wieck et al., 2016). The allergenic and skin sensitizing effects of MIT on humans are known and have resulted in a restriction of their concentration in cosmetics (ECHA, 2014). But concerns are also growing for their use in non-cosmetic products due to their potential impacts to both human (Lidén et al., 2022) and environmental health (Wieck et al., 2016; Kresmann et al., 2018; Nowak-Lange et al., 2022).

The presence of this emerging pollutant in environmental waters has been rarely quantified, with detection reported in wastewater and soil in Poland with a concentration of 1.21 ng/L (Nowak et al., 2020), and in France in surface water and wastewater with concentrations up to 860 ng/L (Paijens, Frère, et al., 2020). Kim & Choi (2022, preprint) investigated trans- and multigenerational effects of the mixture CMIT/MIT (chloro-methylisothiazolinone/methylisothiazolinone, 3:1 ratio) at 7 µg/L on *D. magna*. They documented fitness costs (decrease in the number of juveniles per female, delay in maturation time) of direct and maternal exposure to CMIT/MIT, with suggested transgenerational effects, but also a quick acclimation with enhanced reproductive performances under multigenerational exposure compared to unexposed females. But considering the higher reactivity of CMIT compared to MIT (Collier, Ramsey, Waigh, et al., 1990; Arning et al., 2008), the long-term effects of MIT alone might not be comparable, in particular at relevant concentrations with environmental presence.

Building on previous research presented in the first chapter, this study aimed to investigate possible evolutionary impacts of exposure to MIT on daphnia's fitness. To do so, we studied 6 clonal lines of *D. pulex* exposed both directly and indirectly to MIT over for four generations while recording individual fitness-linked life history traits including survival, reproduction, and growth. Two test concentrations were chosen based on the concentrations of MIT measured in environmental waters (Paijens, Frère, et al., 2020), and range-finding experiments. The objectives of the study were to answer the following: (1) Does MIT exposure induce parental and/or grandparental effects; and, if any, (2) are these effects transmitted even in the absence of direct exposure; (3) do effects of a low and a high concentration compare; (4) is there inter-clonal variation in the response of *D. pulex* to long-term exposure to MIT ?

MATERIAL AND METHODS

Clonal lineages selection and pre-exposure conditions

Five clonal lineages of the species complex *D. pulex/pulicaria* were established from wild populations located in Brittany, France, with an additional clone sourced from Aqualiment© (Table 1). Clonal lineages were acclimatized to laboratory conditions for one year before the start of chemical exposure. Daphnids were reared in charcoal-filtered and dechlorinated tap water under standardised conditions (18±1°C, 16:8 Light:Dark photoperiod) and were fed five times a week with a mixture of *Desmodesmus subspicatus* and *Chlorella vulgaris* microalgae *ad libitum*.

MIT acute toxicity was determined for each clone in the first chapter (Wagner-Deyriès et al., 2023). As a reminder, 48h-LC₅₀ values are reported in Table 2-1. The six clonal lineages were chosen to represent the highest variation in sensitivity available, as inferred from these LC₅₀ values.

Table 2- 1 Location of *D. pulex/pulicaria* populations of the study's clones and corresponding MIT 48h-LC₅₀ values (from Wagner-Deyriès et al., 2023). LC50 = lethal concentration for 50% of the population, CI = credible interval.

Name	Population of origin			Isolation date (month/year)	48h-LC50 (mg/L) median and 95% CI
	Type		Location		
AL0	Commercial strain (Aqualiment©)	-	-	bought in 2016	2.84 (2.66-3.03)
SE5	Natural population	Séné	47.616636, -2.713638	10/2019	1.84 (1.74-1.95)
P16	Aquaculture tanks	Rennes	48.112800, -1.709124	10/2019	1.08 (0.99-1.17)
GO6	Natural population	Goven	47.994867, -1.859119	10/2019	0.78 (0.70-0.87)
SE2	Natural population	Séné	47.616636, -2.713638	10/2019	0.74 (0.66-0.82)
LA0	Natural population	Saint-Judoce	48.359723, -1.951767	11/2019	0.68 (0.60-0.75)

Life-table experiment

Experimental conditions and monitoring

The experiment ran over four clonal generations, each of which was surveyed from birth to the age of 21 days (Figure 2-1). For each generation, test animals were initially reared in 250 mL jars (ca. 25 neonates per jar) from birth to 5 days of age, then individualized in 100 mL beakers filled up to 85% with water. Each daphnid was transferred to a new beaker on day₁₂ and day₁₉, using a pipette with a bevelled edge cut to a minimum opening of 2.5 mm. Daphnids were reared in dechlorinated tap water with a 16:8 L:D photoperiod and fed everyday *ad libitum* with a mixture of *Chlorella vulgaris* and *Desmodesmus subspicatus*. The amount of *C. vulgaris* supplied was doubled on the 12th day of parental (P) generation to compensate for a drop incurred in algal culture concentration, which caused a drop

of reproductive performance and excessive mortality (Figure S2-1). Therefore, the performance of the P generation are not comparable to those of generations F1 - F3, and are not considered in the present study.

In every cohort, survival and offspring production were monitored daily, from day₅ to day₂₁. Newborns and moults were removed every day. Water quality was monitored weekly, using a multiparameter water quality meter (Table S2-1). Additionally, water and air temperature were monitored automatically every 5 minutes with a pen-style thermometer, from 28/07/21 7:02 am to 05/10/21 8:59 am (Figure S2-2).

While life-history traits were monitored on ten individuals isolated in separate beakers, sisters from the same broods were maintained in parallel, as sib groups reared in 1L glass jars. All animals in the experiment originated from synchronous broods of females from the same clonal origin reared in 1L jars. Jar groups were limited to 25 females, with at least 3 jars per condition (clone, generation, treatment, as explained later) in order to ensure sufficient progeny availability throughout the whole experiment. Depending on the number of neonates produced per brood, individuals were randomly collected from the second or the third brood of a given female to start each subsequent generation. However, this rule was not applied to TG.F2 individuals, which originated from the brood released at least 5 days after their mothers (TG.F1 daphnids), and were moved to uncontaminated water (from day 17). This specific treatment aimed to ensure only gametic-mediated exposure of TG.F2 individuals (Figure 2-1).

Treatments

The exposure design is summarized in Figure 1. Control daphnia were kept non-exposed to MIT from generation P (parental) to F3 (great-grand children). Under contaminated conditions, P individuals were exposed to MIT from their day of birth to ensure F1 exposure from the stage of egg cell. In the multigenerational treatment (MG), exposure was continued to F2 and F3, whereas in the transgenerational treatment (TG), mature F1 daphnia were transferred into clean water at the age of 12 days, using 5 successive baths (80 ml volume, 5 seconds per bath). This specific treatment was applied in order to avoid direct exposure of developing embryos intended to establish subsequent generations. With the same objective, and in accordance with *D. pulex* development time (about 3 days at 18°C, Toyota et al., 2016), only neonates produced after a safety period of 5 days in clean water (17 days of maternal age, see Figure 2-1) were used to create filial generations, which were thereafter maintained in clean water (TG.F1 to TG.F3). Therefore, TG.F2 daphnia were exposed at the germline stage at the latest, and TG.F3 daphnia were never directly exposed to MIT. As such, effects observed in TG.F3 animals can be attributed to epigenetic inheritance.

Accounting for some rate of MIT degradation between 2 applications (Reynolds, 1994 from Madsen et al., 2001; Nowak et al., 2020), the 2 nominal concentrations tested (0.8µg/L and 50 µg/L) represent the maximal exposure dose. MIT solution (95% purity; CAS no 2682-20-4) was purchased from Sigma-Aldrich. The lower MIT concentration (LM) was chosen as an environmentally relevant concentration (Paijens, Frère, et al., 2020; Nowak et al., 2020). Range-finding experiments were conducted on batches of 5 individuals from every clone to identify a high MIT concentration (HM) that would not cause 100% mortality at the end of an exposure running from the gametic stage to 21-day-old daphnids (equivalent to MG.F1 exposure which was expected to be the most lethal treatment, see Figure 2-1). In the end, the nominal HM (50 µg/L) corresponds to one tenth of the LC_{20} of the most sensitive clone identified in the acute toxicity chapter (Wagner-Deyriès et al., 2023); compared to LC_{50} , HM is 13.6 – 56.8x lower and LM is 550 – 3550x lower, depending on which clone is considered (Table 2-1).

Life-history traits

To compare individual performances, the following traits were examined from the age of 5 to 21 days: survival, age at first brood expelled, fecundity (total number of live offspring), total number of broods, and total number of dead neonates. Daily survival and reproduction data were also used to determine individual fitness w_i as defined by Fisher (1999):

$$w_i = \sum_{y=0}^{\infty} \lambda^{-y} l_{yi} m_{yi} \quad (1)$$

where the fitness of any individual i is dependent on the value of survival (l_{yi} , either 0 or 1), age-specific reproduction (m_{yi}), intrinsic rate of population growth (λ), and age y . The latter is solved by using the Euler-Lotka equation

$$\sum_{y=0}^{\infty} \lambda^{-y} \bar{l}_y \bar{m}_y = 1 \quad (2)$$

where λ equals to e^r , with r as the rate of population increase (in the Malthusian model), and \bar{l}_y and \bar{m}_y as the mean survival and reproduction rates of the population. A “population” was defined here as a given clonal lineage undergoing a given treatment at a given generation (N=90).

Body size was measured at age 1 and 21 days, and used to compare treatments and lineages from generation F1 to F3. For each treatment group, four neonates were randomly drawn to have their length measured - from the centre of the eye to the base of the spine (Figure S2-3) - and were

then discarded from further assessments (to avoid any artefacts induced by the stress incurred while pictures were taken). The length of any adults reaching the age of 21 day was also recorded (stereomicroscope Leica M125 C and image processing with ImageJ software).

Data analysis

Dataset

Tests were run separately on 6 clonal lineages with 10 replicates (i.e. 10 isolated individuals) per condition, except for 2 replicates that were discarded because they turned out to be males (in LA5.C.F1 and AL0.MG.50.F3). These 2 males were detected after their sisters expelled their first brood, therefore they could not be replaced by females originating from the same clutch. At each generation, 5 treatments were tested including control, multigenerational exposure with MIT nominal concentrations of 50 µg/L (MG.50) or 0.8 µg/L (MG.08), and transgenerational exposure with the same nominal concentrations (TG.50 and TG.08). In total, from generation F1 to F3, we assessed the daily survival and reproduction of N=898 daphnids. Whatever the condition, trait survey only began on day 5, i.e., once daphnia were isolated.

Water temperature monitoring revealed abrupt variations at the end of the experiment (range = 16.25°C to 20.0°C; Figure S2-2) affecting the daphnia that were tested at that time, which showed unusually low reproductive performance. Due to these observations, and the well known impact of temperature on daphnia development and reproduction (Goss & Bunting, 1983; Vanvelk et al., 2021), we decided to remove reproduction data from SE2.TG.50.F3 and all 6 lineages' C.F3. Consequently, control conditions in the F3 were inferred from the corresponding C.F1 performance of each clone replicate. Indeed the reproductive performance of unexposed daphnia in F1 and those with the lowest impact in F3 (TG.08.F3) seemed closer to each other than F2; moreover, performances of TG.08.F3 were also closer to TG.08.F1 than TG.08.F2 (see the total number of offspring and number of broods, Figures S2-14 and S2-15). On the opposite, using the mean trait values of C.F1 and C.F2 would have increased any difference in trait values between controls and exposed daphnids. This strategy was thought to be as neutral as possible, in a context where the whole experiment could not be restarted from scratch.

Statistical analysis

All analyses were conducted using R version 4.2.2 (R Core Team, 2022), with dataset modified in control F3 as explained above (see 2.4.1. section). To test the effects of MIT treatments over three

clonal generations on life-history traits, generalized linear mixed-effect models (GLMM) were fitted with the `glmer` function of the package 'lme4' version 1.1-31 (Bates et al., 2015). Exposure type (C, MG.08, MG.50, TG.08, TG.50) and generation (F1, F2, F3) were included as fixed effects, and clonal lineage was included as random effect (one model per trait, same model structure for all traits). For daily survival, the age was also added as a fixed-effect, and variance among lineage was included in the coefficient terms but not in the intercept. P-values were calculated with a likelihood-ratio test, by comparing the likelihood of full versus restricted models (Table 2-2). Post-hoc comparisons of model estimates - with clones included as a fixed effect variable - were conducted with least-squares method with the 'emmeans' package version 1.8.4-1 (Lenth, 2016).

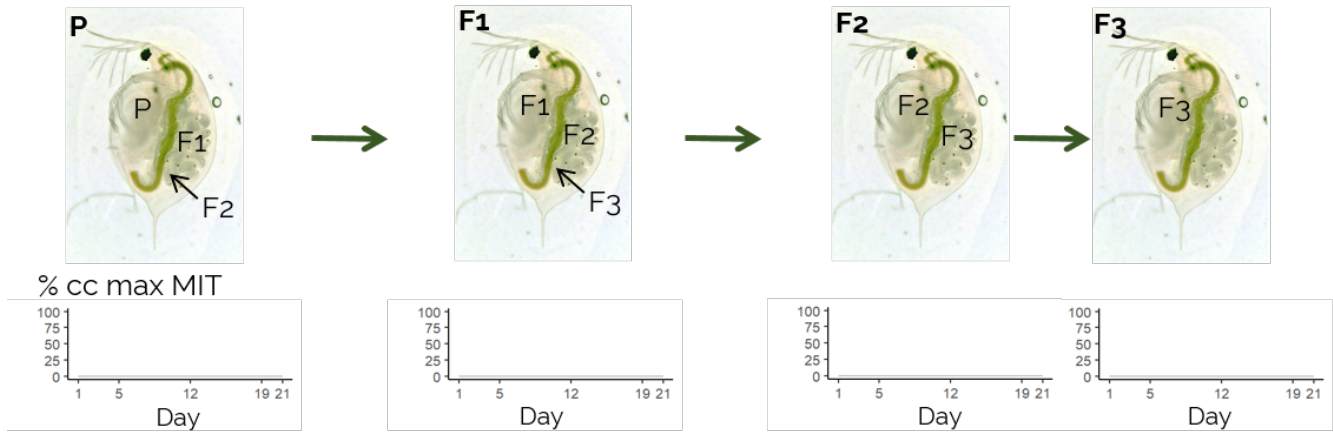
MIT effective concentration

MIT effective concentrations were measured using an ultra-performance liquid chromatography triple-quadrupole mass spectrometer (UPLC-MS/MS, XEVO TQD; Waters). Samples from MG08F3 and MG50F3 were collected in 3 individual beakers and 3 breeding jars at treatment renewal, 3 days later and 7 days later, and then pooled per jar type and day after renewal, and filtered (0.22 μm glass fiber/cellulose acetate, Phenomenex). MIT-d3 (2-methyl-d3-4-isothiazolin-3-one hydrochloride, analytical standard, CAS no 1329509-49-0, Sigma-Aldrich) was added as internal isotopically labeled standard for MIT. Injections of samples were repeated 10 times. For the separation, a Waters Acquity UPLC BEH C18 column (50mm x 2.1 mm inner diameter, 1.7 μm particle size) was used, with the column temperature maintained at 30°C. The mobile phase (solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in acetonitrile, UPLC-grade solvents) flow rate was set at 0.7 mL.min⁻¹, and injection volume was set at 5 μL . The mobile phase was initially started at 5% B at 0 min and increased to 15% B within 2.9 min, and held at 5% until 3.5 min. The MS was operated in the positive ESI mode with a capillary voltage of 3 kV and a cone voltage of 30 V. Acquisition was achieved in multiple reaction monitoring mode (MRM), with a time scan of 0.25 s. Parameters for each transition are presented in supporting table S2-2. Data acquisition and integration were respectively conducted with MassLynx® and TargetLynx® software (v4.2; Waters). The comparison of predicted concentrations between jar's type (individual beaker or breeding jar) and time after water renewal was conducted in R, with tests of Kruskal-Wallis followed by Dunn's tests if the first was significant.

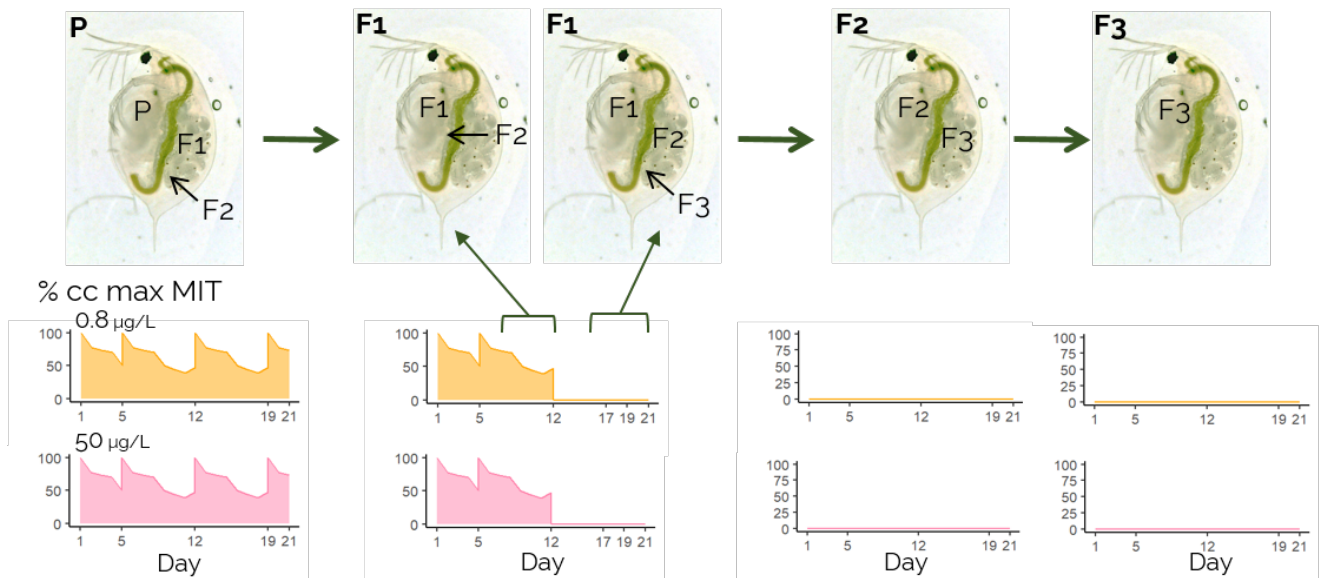
The same method was applied with a UPLC coupled with a quadrupole time-of-flight mass spectrometer (XEVO G2XS QToF, Waters) with 3 individual beakers sampled every day for 7 days after (4 injections of 3 replicates). Because of the lower sensitivity of the QToF, absolute concentrations' predictions were not accounted for, but the relative concentrations in MG50 samples were used to identify a pattern of decrease in MIT concentration. Indeed, in both experiments, the concentration

seemed to decreased to about half the initial concentration after 7 days in experimental conditions. This pattern was extrapolated to the whole trial (Figure 2-1). Further information can be found in supplementary material.

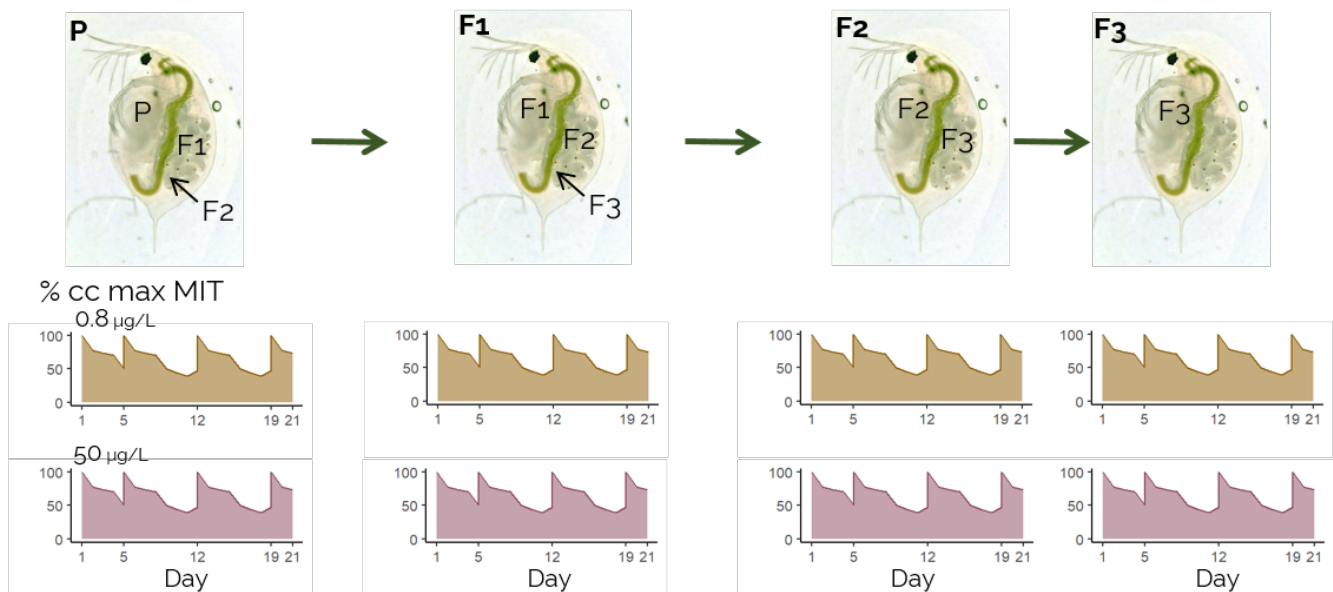
CONTROL



P AND F1-ONLY EXPOSURE



MULTIGENERATIONAL EXPOSURE



CONTROL

Control daphnia were never exposed to MIT

MIT EXPOSURE

08 = maximal nominal concentration 0.8 µg/L, Low MIT (**LM**)

50 = maximal nominal concentration 50 µg/L, High MIT (**HM**)

P AND F1-ONLY EXPOSURE

TG P = direct exposure from neonate stage to 21 days of age

TG F1 = direct exposure from germ cell stage (in P) to 12-days old, and unexposed from 12-days old until 21-days old

TG F2 = germ cell stage exposure (in F1) only, not directly exposed from their deposition in the F1 brood pouch, nor at later stages

TG F3 = unexposed

MULTIGENERATIONAL EXPOSURE

MG P = direct exposure from neonate stage to 21 days of age

MG F1 = direct exposure from germ cell stage (in P) to 21 days of age

MG F2 = direct exposure from germ cell stage (in P and F1) to 21 days of age

MG F3 = direct exposure from germ cell stage (in F1 and F2) to 21 days of age

Figure 2- 1 Schematic overview of the experimental design. TG = transgenerational (note that true transgenerational exposure only concerns TG.F3 daphnids), MG = multigenerational. The temporal evolution of MIT concentration is depicted by the bottom curves and areas: effective concentration shown as percent of its maximal value (0.8 or 50 µg/L), each peak corresponding to a new contamination. Experiments were run simultaneously on 6 clones of *D. pulex*, with 10 replicates for each clone in each treatment (N=898).

RESULTS

MIT effective concentration

The mean effective concentrations of MIT are displayed in Table S2-3 and Table S2-4. The correlation coefficient of the calibration curve (Figure S2-6a) for HM nominal concentration is acceptable and confirmed as linear ($R^2 = 0.996$), but this was not the case for LM nominal concentration ($R^2 = 0.738$), probably because values were too close to the limit of quantification (Figure S2-6c). The pattern of decrease in MIT concentration over time for the different treatments was extrapolated from measured values at HM concentrations, and is displayed in Figure 2-1. Globally, results suggest that MIT concentration was halved after 7 days of interactions with daphnids, fresh microalgae, and their associated microorganisms ($H = 40.6$, $p\text{-value} < 0.001$, HM dataset). Therefore, the design implied pulse exposure conditions, with weekly peaks corresponding to recontaminations (i.e. changes of media). Moreover, the maximum values of effective concentrations were probably lower than nominal concentrations, with initial measured values of 24 $\mu\text{g/L}$ instead of 50 $\mu\text{g/L}$ (Table S2-3) and 0.28 $\mu\text{g/L}$ instead of 0.8 $\mu\text{g/L}$ (Table S2-4). No significant difference was found in effective concentrations between individual beakers and breeding jars ($H = 3.38$, $p\text{-value} = 0.184$ in HM concentration dataset, and $H = 0.549$, $p\text{-value} = 0.76$ in LM concentration dataset).

Global effects

In order to assess global trends in trait variation, data were firstly analysed without accounting for lineage origin.

Effects on survival

Even though concentrations were initially selected to be sub-lethal, as extrapolated from acute toxicity data (48h dose-response curves), survival was impaired by the direct exposure to MIT from the start of the survey, especially in the F1 generation (see survival curves Figure 2-2, Figure S2-12, Table 2-2, and Table 2-3).

Within-generations survival patterns are displayed in Figure 2-2. In F1, the majority of HM-exposed animals did not survive up to 21 days ($p\text{-value} < 0.05$, Table 2-3 and Figure S2-12), whereas LM-exposed organisms did not differ from controls (NS $p\text{-value}$). It is to be recalled that in F1, TG and MG represent very similar treatments (Figure 2-1). In F2, only MG.50-exposed daphnia were highly impacted (Table 2-3 and Figure S2-12). In the last generation, the survival curves of MG.08 and MG.50 individuals almost overlapped (NS $p\text{-value}$, Table 2-3), reflecting improved survival at high

concentration (MG.50), as compared to F2, with a mean 21-day survival of 16.8 days against 9.3 days in F2.

Overall, direct exposure to MIT (MG) affected negatively the survival of daphnia, however opposite trends were depicted between the two studied concentrations (HM vs LM), with, from F1 to F3, a worsening of effects at low concentration and a clear improvement in tolerance at high concentration.

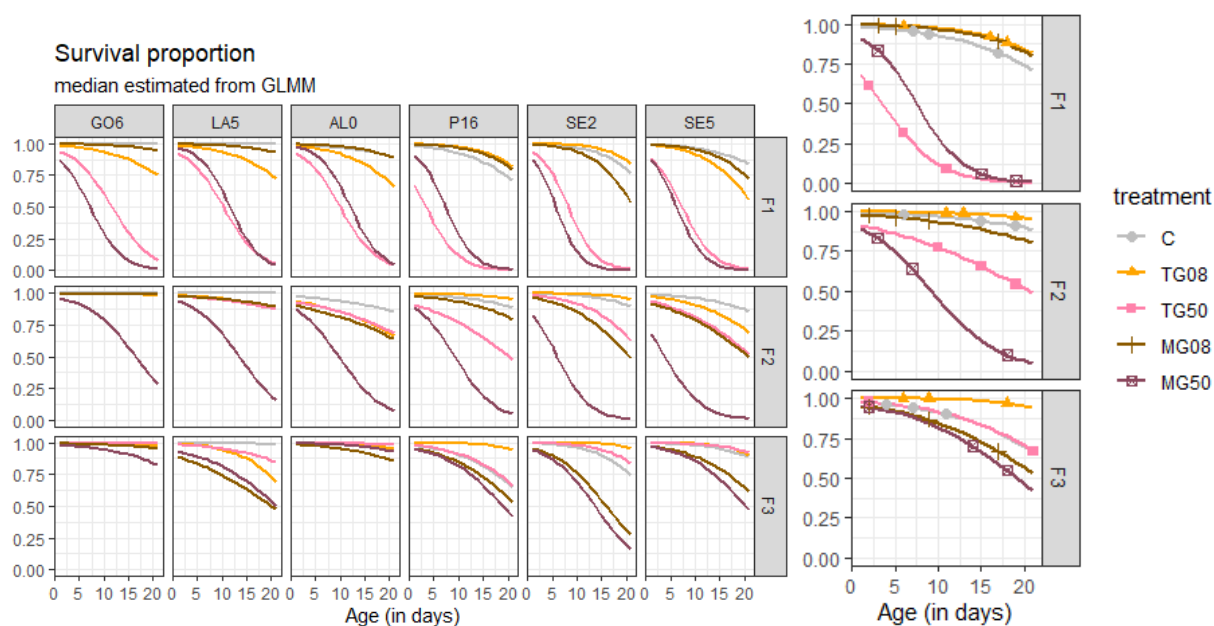


Figure 2- 2 Median survival proportion estimates from GLMMs, with clonal lineages plotted separately (left panel) as well as a global conditioned median (right panel). The 95% prediction intervals are not displayed for ease of viewing, but are presented in Figure S2-13.

Effects on size at birth and at maturity

Over the course of the survey, the global analysis revealed significant effects of chemical treatment, generation, and of their interaction on both body size measurements (p-value < 0.001, Table 2-2).

Multiple comparison tests showed that at birth, MG.50.F2 neonates were significantly smaller than under any other condition, excepted TG.50.F2, which on its side was intermediate and did not differ from various other conditions (Table 2-3 and Figure S2-18). While exposure over one single generation (P) – exposed from the neonate stage - did not affect size at birth of their progeny (F1), it did have a negative effect on this trait in the following generation (F2), whatever the concentration applied in F1 (TG.08.F2 = TG.50.F2 < C.F2, Table 2-3). This effect was only transient and disappeared in the grand progeny (TG.08.F3 = TG.50.F3 = C.F3). The same trend was observed in MG.50 exposed

organisms, with a strong reduction of size at birth in F2, followed by a return to control-like values in F3. It is to be noted that MG.08 exposure conditions had no significant effect on this trait.

Regarding body size at the age of 21 days, no significant departure from control conditions could be detected under any treatment (chemical or generation). The significant effects of treatment revealed by the global analysis was due to inter-generational variations such as the larger size of control individuals in F2 compared to F3 (Table 2-3 and Figure S2-19).

Effects on traits related to reproduction (age at first brood, lifetime number of neonates, lifetime number of broods, and number of dead neonates)

For all life-history traits taken separately, the effects of chemical treatment, generation and their interaction were significant (p -value < 0.001 , Table 2-2), which was mostly due to differences between generations (Table 2-3, Figures S2-14, S2-15, S2-16, and S2-17). In general, F2 individuals displayed better reproductive performance than in other generations (Table 2-3), in particular with larger clutch size (mean values for breeding females: F1 = 65, F2 = 86, and F3 = 54). On the contrary, F3 individuals had small clutch size, they expulsed fewer broods (mean values for breeding females: F1 = 4.2, F2 = 4.3, and F3 = 3.7), and had more aborted neonates (mean values for breeding females: F1 = 3, F2 = 2, and F3 = 7). Individuals mostly matured at the same age between generations (mean values of age at first brood release: F1 = 10.7 days, F2 = 10.6 days, and F3 = 11.2 days). The general low reproduction performance of F3 indicate a discrepancy between F3 and other generations (Table 2-3).

Within each generation, no major differences were observed between treatments for the numbers of clutches, the number of progeny, nor the age at first brood (Table 2-3). However, in F1, while only a few females bred under HM exposure, they had a mean number of dead offspring 3 times lower than their counterparts ($p < 0.05$, Figure SX). In F2, MG.50-exposed breeding individuals still expulsed fewer dead offspring than under other treatments (except controls), and this difference was not maintained in the next generation. Overall, the very few clues regarding potential effects of treatment on the reproductive performance of daphnids across generations hint towards an improved reproduction of the females exposed to HM from the embryonic stage and who managed to reach adulthood; however, this effect disappeared in F3.

Effects on individual fitness (w)

A global picture of MIT effects on daphnia is provided by individual fitness, as estimated from the set of fitness-related traits measured throughout the experiment. This global pattern was consistent with that reflected by each life-history trait taken separately (Figure X). Fitness was

significantly different between generations (p-value < 0.001, Table 2-2), MIT exposure (p-value < 0.001), and their interaction (p-value < 0.001). Roughly, the generational effect separated individuals surviving long enough to reproduce (e.g. controls) from those who did not (e.g. most F1.MG.50 individuals). Hence, individual fitness was mainly determined by survival in this experiment.

In the first generation, individuals directly exposed to HM had a median w of 0.0 (MG.50 = TG.50), while individuals exposed to LM had a median w of 1.0 (MG.08 = TG.08), which was not different from that of controls (NS p-value, Table 2-3). In the next generation, the mean fitness of daphnia exposed to HM only at the germ cell stage (F2.TG.50) was not different from the fitness of daphnids exposed to LM and of controls (NS p-value, Table 2-3); daphnia continuously exposed to HM however were still highly affected (NS p-value, Table 2-3). In the third generation, the comparison of means did not reveal statistically significant differences between any treatments (Table 2-3). Nevertheless, the distribution of fitness in F3 generation was affected by the mode of exposure, i.e., multi- vs transgenerational, rather than by the concentration: F3.MG.08&50 individuals were more similar to each other than to daphnids not directly exposed (F3.TG.08&50) (see Figure 2-3 for w distributions and Figure S2-10 for w quantile-quantile plots). One obvious difference between MG and TG effects is the larger proportion of dead or non-breeding F3 females ($w = 0$) in MG. Additionally, within breeding females ($w > 0$ in Figure X), fitness exhibited a wider distribution under MG (08 from 0.2 to 4.2; 50 from 0.2 to 3.2) than under TG conditions (08 from 0.3 to 1.9; 50 from 0.1 to 2.0).

Focusing on breeding females, direct exposure to HM in F1 and F2 (F1.TG.50, F1.MG.50, and F2.MG.50, Figure 2-4) increased individual fitness significantly (p-value < 0.05, Table 2-3). In the last generation (F3), although no significant departure was detected across treatments (NS p-value, Table 2-3), as already mentioned for the full datasets, fitness distributions remained more homogeneous within treatment modes (continuous, indirect) than between them (Figure 2-4 and Figure S2-11). These results are consistent with those reflecting reproductive performance (see section 2.3 above). They also reflect how this specific measure of individual fitness highlighted subtle differences between performances that were not detected with global indicators. The absence of differences between values of fitness of breeding females of F1.TG.50 and F1.MG.50 (NS p-value, Table 3) indicates that the transfer of daphnids to uncontaminated water after maturity did not improve their individual fitness.

Overall, the fitness of daphnia directly exposed to MIT was negatively impacted, either from the start (HM) or after a few generations (LM), while no effect was noticed following ancestral-only exposure.

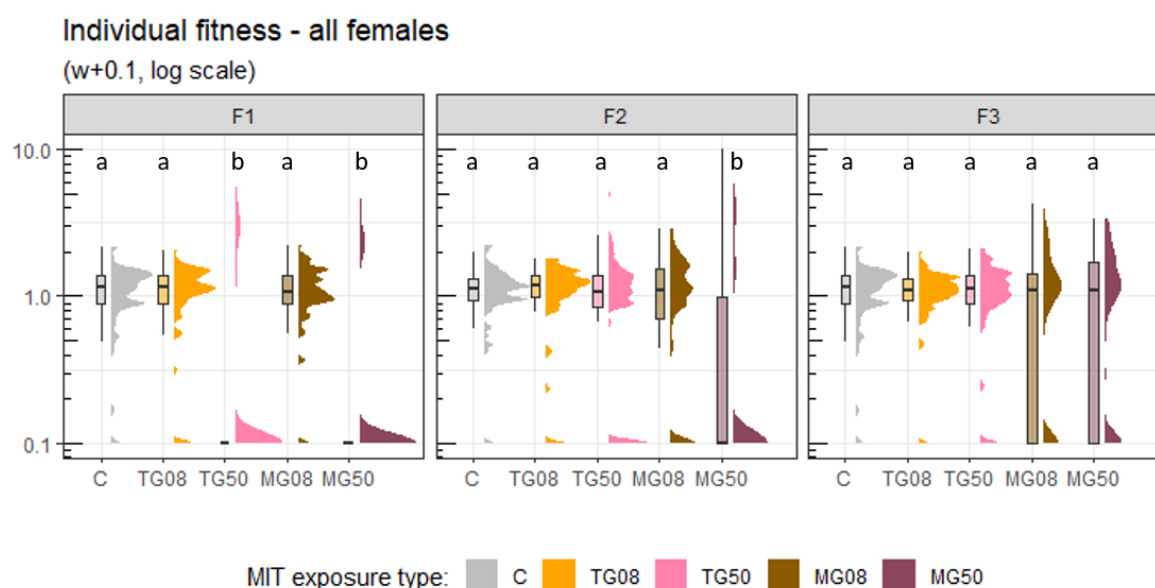


Figure 2- 3 Individual fitness (including breeding and non-breeding females), per generation and exposure type to MIT (C: controls; TG: transgenerational; MG: multigenerational; 08: maximal dose 0.8 $\mu\text{g/L}$; 50: maximal dose 50 $\mu\text{g/L}$), all lineages included (n=60). Data for C.F3 were substituted by C.F1 (see material and methods 2.4.1). Top letters represent groups contrasted by pairwise least-squares means comparison, with p-values adjusted with the Tukey method and a confidence level used of 0.95.

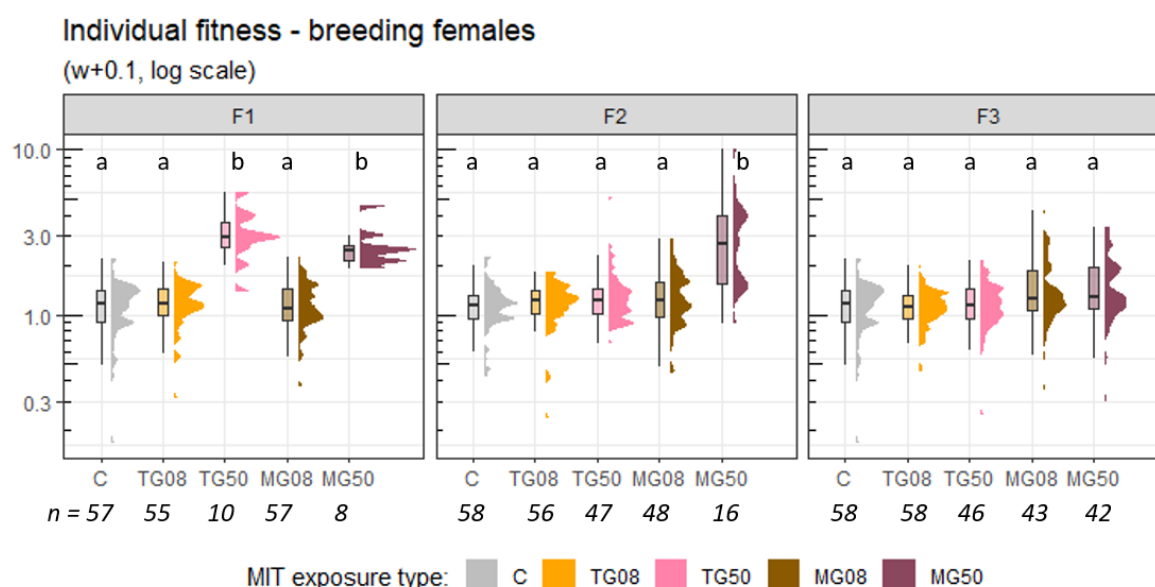


Figure 2- 4 Individual fitness of breeding females only, per generation and exposure type to MIT (C: controls; TG: transgenerational; MG: multigenerational; 08: maximal dose 0.8 $\mu\text{g/L}$; 50: maximal dose 50 $\mu\text{g/L}$), all lineages included (sample size indicated below each boxplot). Data for C.F3 were substituted by C.F1 (see material and methods 2.4.1). Top letters represent groups contrasted by pairwise least-squares means comparison, with p-values adjusted with the Tukey method and a confidence level used of 0.95.

Effect of lineage

All studied traits showed heterogeneity at the lineage level (see p-values associated with lineage effects in Table 2-2). Moreover, each studied line presented its own multigenerational norm of reaction towards chemical stress, in terms of survival as well as reproductive performance (supplementary Figure S2-13, S2-21, S2-22, S2-23, and S2-24). As a consequence, the fitness response also diverged among clones, suggesting differences in adaptive potential towards MIT-induced chemical pressure. This divergence was mostly detectable in F2 and F3, whereas F1 individuals showed very similar dose-response curves (with the exception of AL0.MG.50-F1 daphnids, whose fitness was much less reduced than in any other line, Figure 2-5 top). In F2, cumulative exposure conditions (MG) triggered heterogeneous effects on fitness among lines, with 3 main types of dose-response relationship: GO6, characterized by a loss of sensitivity to HM (compared to F1), AL0-LA5-P16-SE2, sensitive to HM only, and SE5, the most sensitive, as affected at both LM and HM (Figure X). In F3, cumulative exposure conditions (MG) induced wider discrepancies between lines, with 3 groups, AL0-GO6, who appeared perfectly acclimated to low and high concentrations, P16-SE5, showing persistent sensitivity to HM, and LA5-SE2, who started to become sensitive to LM exposure only. In the end, 5 out of the 6 lineages displayed a loss of sensitivity to HM exposure, either from the 3rd generation exposed (F2) in the case of GO6, or from the 4th generation exposed (F3) for AL0-LA5-P16-SE2.

By contrast to MG exposure, all 6 clones responded in a very similar way to TG conditions, i.e., without fitness impairment, to the noticeable exception of P16.TG.50.F2 individuals (see specific fitness decrease in Figure 2-5).

Compared with the ranking of lineages with respect to their 48h-LC₅₀ (Table 2-1), individuals from AL0 were also in the most tolerant clones and those from LA5-SE2-P16 formed the most sensitive group of clones. However, GO6 and SE5 were respectively more tolerant and sensitive than expected based on their 48h-LC₅₀ values.

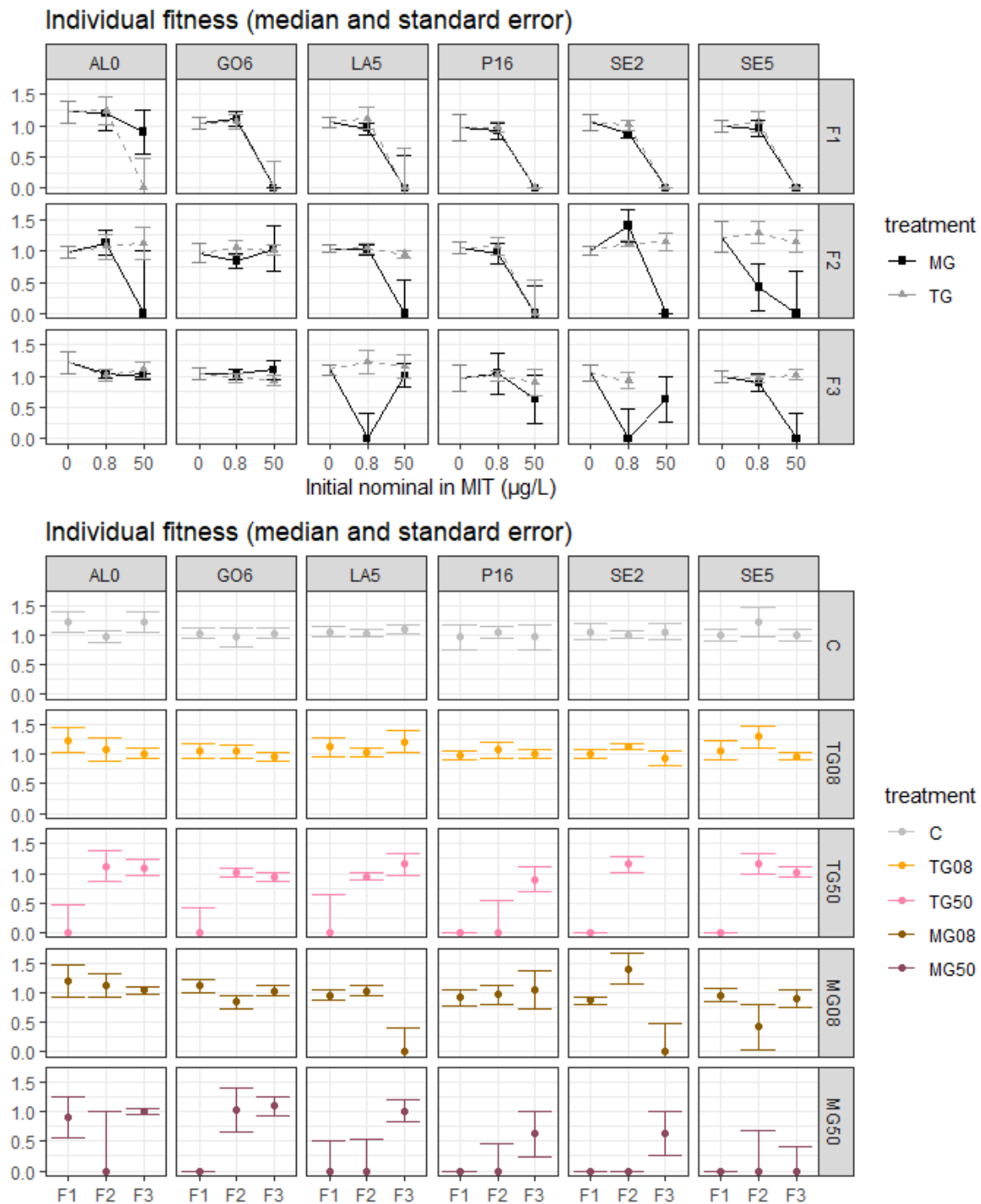


Figure 2- 5 Individual fitness (including breeding and non-breeding females), per generation, clonal lineage, and exposure to MIT (C: controls; TG: transgenerational; MG: multigenerational; 08: maximal dose 0.8 $\mu\text{g/L}$; 50: maximal dose 50 $\mu\text{g/L}$), all lineages included (n=10).

Table 2- 2 Likelihood ratio test results of fixed effects (treatment, generation, and genotype for all traits, and age in the case of survival) and clonal lineage of the general linear mixed effect models (GLMM) per life history traits, all generations included. All models include random variation amongst technical replicates nested in clonal lineage effect.

Response variable		Chi-Square	Df	Pr(>Chisq)
Survival	Fixed effects			
	Treatment	1509	24	<0.001
	Generation	1459.8	20	<0.001
	Age	260.72	15	<0.001
	Treatment X Generation	1401.3	16	<0.001
	Treatment X Age	130.73	12	<0.001
	Age X Generation	155.38	10	<0.001
	Age X Treatment X Generation	1508.6	22	<0.001
	Random effect			
Age at first brood	Lineage	24605	24	<0.001
	Fixed effects			
	Treatment	453.9	12	<0.001
	Generation	174.69	10	<0.001
	Treatment X Generation	69.07	8	<0.001
	Random effect			
	Lineage	210.16	1	<0.001
Total number of neonates per female	Fixed effects			
	Treatment	41.35	12	<0.001
	Generation	149.92	10	<0.001
	Treatment X Generation	33.451	8	<0.001
	Random effect			
	Lineage	58.984	1	<0.001
Total number of broods per female	Fixed effects			
	Treatment	195.05	12	<0.001
	Generation	179.99	10	<0.001
	Treatment X Generation	164.09	8	<0.001
	Random effect			
	Lineage	50.849	1	<0.001
Total number of dead neonates per female	Fixed effects			
	Treatment	40.514	12	<0.001
	Generation	112.15	10	<0.001
	Treatment X Generation	22.593	8	0.004
	Random effect			
	Lineage	160.53	1	<0.001
Individual fitness	Fixed effects			
	Treatment	136.36	12	<0.001
	Generation	356.95	10	<0.001
	Treatment X Generation	673.29	8	<0.001
	Random effect			
	Lineage	335.46	1	<0.001
Size at day 1	Fixed effects			
	Treatment	72.738	12	<0.001
	Generation	122.63	10	<0.001
	Treatment X Generation	38.063	8	<0.001
	Random effect			
	Lineage	26.141	1	<0.001
Size at day 21	Fixed effects			
	Treatment	42.847	12	<0.001
	Generation	89.034	10	<0.001
	Treatment X Generation	41.332	8	<0.001
	Random effect			
	Lineage	329.02	1	<0.001

Table 2- 3 Pairwise least-squares means comparison, with p-values adjusted with the Tukey method and a confidence level of 0.95, results for life history traits within each generation, all clonal lineages merged or separated. NA missing data, ns not significantly different, *p-value<0.05, + increase, - decrease (i.e. “expo1 vs expo2 = - *” means trait for expo1 is significantly decreased compared to the same trait for expo2).

	Number of neonates	Number of broods	Age at first brood	Number of dead neonates	Size at 1-day- old	Size at 21-days- old	Number of days survived	Individual fitness
F1								
Effect of direct exposure (exposed from cell stage vs never exposed)								
TG08 vs C	ns	ns	ns	ns	ns	ns	ns	ns
GO6	ns	ns	ns	ns	ns	ns	ns	ns
LA5	ns	ns	ns	ns	ns	ns	ns	ns
AL0	ns	ns	ns	ns	ns	ns	ns	ns
P16	ns	ns	ns	ns	ns	ns	ns	ns
SE2	ns	ns	ns	ns	ns	ns	ns	ns
SE5	ns	ns	ns	ns	ns	ns	ns	ns
TG50 vs C	ns	ns	ns	-*	ns	ns	-*	-*
GO6	ns	ns	ns	ns	ns	-*	-*	ns
LA5	ns	ns	ns	ns	ns	ns	-*	ns
AL0	ns	ns	ns	ns	ns	ns	-*	ns
P16	NA	NA	NA	NA	ns	NA	-*	-*
SE2	NA	NA	NA	NA	ns	NA	-*	-*
SE5	NA	NA	NA	NA	ns	NA	-*	-*
MG08 vs C	ns	ns	ns	ns	ns	ns	ns	ns
GO6	ns	ns	ns	ns	ns	ns	ns	ns
LA5	ns	ns	ns	ns	ns	ns	ns	ns
AL0	ns	ns	ns	ns	ns	ns	ns	ns
P16	ns	ns	ns	ns	ns	ns	ns	ns
SE2	-*	ns	ns	ns	ns	ns	ns	ns
SE5	ns	ns	ns	ns	ns	ns	ns	ns
MG50 vs C	ns	ns	ns	-*	ns	ns	-*	-*
GO6	NA	NA	NA	NA	ns	NA	-*	-*
LA5	ns	ns	ns	-*	ns	ns	-*	ns
AL0	ns	ns	ns	ns	ns	ns	ns	ns
P16	NA	NA	NA	NA	ns	NA	-*	-*
SE2	NA	NA	NA	NA	ns	NA	-*	-*
SE5	NA	NA	NA	NA	ns	NA	-*	-*
Effect of dose (50 vs 0.8 µg/L)								
TG50 vs TG08	ns	ns	ns	-*	ns	ns	-*	-*
GO6	ns	ns	ns	ns	ns	ns	ns	ns
LA5	ns	ns	ns	ns	ns	ns	ns	ns
AL0	ns	ns	ns	ns	ns	ns	ns	ns
P16	NA	NA	NA	NA	ns	NA	-*	-*
SE2	NA	NA	NA	NA	ns	NA	-*	-*
SE5	NA	NA	NA	NA	ns	NA	-*	-*
MG50 vs MG08	ns	ns	ns	-*	ns	ns	-*	-*
GO6	NA	NA	NA	NA	ns	NA	-*	-*
LA5	ns	ns	ns	ns	ns	ns	-*	ns
AL0	ns	ns	ns	ns	ns	ns	ns	ns
P16	NA	NA	NA	NA	ns	NA	-*	-*
SE2	NA	NA	NA	NA	ns	NA	-*	-*

Table 2- 3 Pairwise least-squares means comparison, with p-values adjusted with the Tukey method and a confidence level of 0.95, results for life history traits within each generation, all clonal lineages merged or separated. NA missing data, ns not significantly different, *p-value<0.05, + increase, - decrease (i.e. “expo1 vs expo2 = - *” means trait for expo1 is significantly decreased compared to the same trait for expo2).

	SE5	Number of neonates NA	Number of broods NA	Age at first brood NA	Number of dead neonates NA	Size at 1-day- old ns	Size at 21-days- old NA	Number of days survived -*	Individual fitness -*
Effect of continued exposure vs interrupted exposure (MG vs TG)									
MG08 vs TG08	ns	ns	ns	ns	ns	ns	ns	ns	ns
GO6	ns	ns	ns	ns	ns	ns	ns	ns	ns
LA5	ns	ns	ns	ns	ns	ns	ns	ns	ns
AL0	ns	ns	ns	ns	ns	ns	ns	ns	ns
P16	ns	ns	ns	ns	ns	ns	ns	ns	ns
SE2	ns	ns	ns	ns	ns	ns	ns	ns	ns
SE5	ns	ns	ns	ns	ns	ns	ns	ns	ns
MG50 vs TG50	ns	ns	ns	ns	ns	ns	ns	ns	ns
GO6	NA	NA	NA	NA	ns	NA	ns	ns	ns
LA5	ns	ns	ns	ns	ns	ns	ns	ns	ns
AL0	ns	ns	ns	ns	ns	ns	ns	ns	ns
P16	NA	NA	NA	NA	ns	NA	ns	ns	ns
SE2	NA	NA	NA	NA	ns	NA	ns	ns	ns
SE5	NA	NA	NA	NA	ns	NA	ns	ns	ns
F2									
Effect of direct exposure for 3 generations (since P)									
MG08 vs C	ns	ns	ns	ns	-*	ns	ns	ns	ns
GO6	ns	ns	ns	ns	ns	ns	ns	ns	ns
LA5	ns	ns	ns	ns	ns	ns	ns	ns	ns
AL0	ns	ns	ns	ns	ns	ns	ns	ns	ns
P16	ns	ns	ns	ns	-*	ns	ns	ns	ns
SE2	ns	ns	ns	ns	-*	ns	ns	ns	ns
SE5	ns	ns	ns	ns	ns	ns	ns	ns	ns
MG50 vs C	ns	ns	ns	ns	-*	ns	-*	-*	-*
GO6	ns	ns	ns	ns	ns	ns	ns	ns	ns
LA5	ns	ns	ns	ns	ns	ns	-*	ns	ns
AL0	ns	ns	ns	ns	ns	ns	-*	-*	ns
P16	ns	ns	ns	ns	-*	ns	-*	ns	ns
SE2	NA	NA	NA	NA	-*	NA	-*	-*	ns
SE5	ns	ns	ns	ns	-*	ns	-*	ns	ns
Effect of germinal cell exposure vs never exposed									
TG08 vs C	ns	ns	ns	ns	-*	-*	ns	ns	ns
GO6	ns	ns	ns	ns	ns	ns	ns	ns	ns
LA5	ns	ns	ns	ns	ns	ns	ns	ns	ns
AL0	ns	ns	ns	ns	ns	ns	ns	ns	ns
P16	ns	ns	ns	ns	-*	ns	ns	ns	ns
SE2	ns	ns	ns	ns	-*	ns	ns	ns	ns
SE5	ns	ns	ns	ns	-*	ns	ns	ns	ns
TG50 vs C	ns	ns	ns	ns	-*	ns	ns	ns	ns
GO6	ns	ns	ns	ns	ns	ns	ns	ns	ns
LA5	ns	ns	ns	ns	ns	ns	ns	ns	ns
AL0	ns	ns	ns	ns	ns	ns	ns	ns	ns

Table 2- 3 Pairwise least-squares means comparison, with p-values adjusted with the Tukey method and a confidence level of 0.95, results for life history traits within each generation, all clonal lineages merged or separated. NA missing data, ns not significantly different, *p-value<0.05, + increase, - decrease (i.e. “expo1 vs expo2 = - *” means trait for expo1 is significantly decreased compared to the same trait for expo2).

		Number of neonates	Number of broods	Age at first brood	Number of dead neonates	Size at 1-day- old	Size at 21-days- old	Number of days survived	Individual fitness
	P16	ns	ns	ns	ns	-*	ns	-*	ns
	SE2	ns	ns	ns	ns	-*	ns	ns	ns
	SE5	ns	ns	ns	ns	-*	ns	ns	ns
Effect of lifetime exposure vs germinal cell exposure									
MG08 vs TG08		ns	ns	ns	ns	ns	ns	ns	ns
	GO6	ns	ns	ns	ns	ns	ns	ns	ns
	LA5	ns	ns	ns	ns	ns	ns	ns	ns
	AL0	ns	ns	ns	ns	ns	ns	ns	ns
	P16	ns	ns	ns	ns	ns	ns	ns	ns
	SE2	ns	ns	ns	ns	ns	ns	ns	ns
	SE5	ns	ns	ns	ns	ns	ns	ns	ns
MG50 vs TG50		ns	ns	ns	-*	ns	ns	-*	-*
	GO6	ns	ns	ns	ns	ns	ns	ns	ns
	LA5	ns	ns	ns	ns	ns	ns	-*	ns
	AL0	ns	ns	ns	ns	ns	ns	-*	ns
	P16	ns	ns	ns	ns	ns	ns	ns	ns
	SE2	NA	NA	NA	NA	ns	NA	-*	-*
	SE5	ns	ns	ns	ns	ns	ns	-*	ns
Effect of dose (50 vs 0.8 µg/L)									
TG50 vs TG08		ns	ns	ns	ns	ns	+*	ns	ns
	GO6	ns	ns	ns	ns	ns	ns	ns	ns
	LA5	ns	ns	ns	ns	ns	ns	ns	ns
	AL0	ns	ns	ns	ns	ns	ns	ns	ns
	P16	ns	ns	ns	+*	ns	ns	-*	ns
	SE2	ns	ns	ns	ns	ns	ns	ns	ns
	SE5	ns	ns	ns	ns	ns	ns	ns	ns
MG50 vs MG08		ns	ns	ns	-*	-*	ns	-*	-*
	GO6	ns	ns	ns	ns	ns	ns	ns	ns
	LA5	ns	ns	ns	ns	ns	ns	-*	ns
	AL0	ns	ns	ns	ns	ns	ns	-*	ns
	P16	ns	ns	ns	ns	ns	ns	-*	ns
	SE2	NA	NA	NA	NA	-*	NA	-*	-*
	SE5	ns	ns	ns	ns	ns	ns	ns	ns
F3									
Effect of dose (50 vs 0.8 µg/L)									
TG50 vs TG08		ns	ns	ns	ns	ns	ns	ns	ns
	GO6	ns	ns	ns	ns	ns	ns	ns	ns
	LA5	ns	ns	ns	ns	ns	ns	ns	ns
	AL0	ns	ns	ns	ns	ns	ns	ns	ns
	P16	ns	ns	ns	ns	ns	ns	ns	ns
	SE2	NA	NA	NA	NA	NA	NA	NA	NA
	SE5	ns	ns	ns	ns	ns	ns	ns	ns
MG50 vs MG08		ns	ns	ns	ns	ns	ns	ns	ns
	GO6	ns	ns	ns	ns	ns	ns	ns	ns

Table 2- 3 Pairwise least-squares means comparison, with p-values adjusted with the Tukey method and a confidence level of 0.95, results for life history traits within each generation, all clonal lineages merged or separated. NA missing data, *ns* not significantly different, *p-value<0.05, + increase, - decrease (i.e. “expo1 vs expo2 = - *” means trait for expo1 is significantly decreased compared to the same trait for expo2).

		Number of neonates	Number of broods	Age at first brood	Number of dead neonates	Size at 1-day- old	Size at 21-days- old	Number of days survived	Individual fitness
	LA5	ns	ns	ns	ns	ns	NA	ns	ns
	AL0	ns	ns	ns	ns	ns	ns	ns	ns
	P16	ns	ns	ns	ns	ns	ns	ns	ns
	SE2	ns	ns	ns	ns	ns	ns	ns	ns
	SE5	ns	ns	ns	ns	ns	ns	ns	ns
Transgenerational effect (with controls from F1)									
TG08 vs C		ns	-*	ns	ns	ns	ns	ns	ns
	GO6	ns	ns	ns	ns	ns	ns	ns	ns
	LA5	ns	ns	ns	ns	ns	ns	ns	ns
	AL0	ns	ns	ns	ns	ns	ns	ns	ns
	P16	ns	ns	ns	ns	ns	+	ns	ns
	SE2	ns	ns	ns	ns	ns	ns	ns	ns
	SE5	ns	ns	ns	ns	ns	ns	ns	ns
TG50 vs C		ns	-*	+	+	ns	ns	ns	ns
	GO6	ns	ns	ns	ns	ns	ns	ns	ns
	LA5	ns	ns	ns	ns	ns	ns	ns	ns
	AL0	ns	ns	ns	ns	ns	ns	ns	ns
	P16	ns	ns	ns	ns	ns	ns	ns	ns
	SE2	NA	NA	NA	NA	NA	NA	NA	NA
	SE5	ns	ns	ns	ns	ns	ns	ns	ns
Effect of direct exposed for 4 generations (with controls from F1)									
MG08 vs C		ns	ns	+	ns	+	ns	ns	ns
	GO6	ns	ns	ns	ns	ns	ns	ns	ns
	LA5	ns	ns	ns	ns	ns	NA	ns	ns
	AL0	ns	ns	ns	ns	ns	ns	ns	ns
	P16	ns	ns	ns	ns	ns	ns	ns	ns
	SE2	ns	ns	ns	ns	ns	ns	ns	ns
	SE5	ns	ns	ns	ns	ns	ns	ns	ns
MG50 vs C		ns	ns	ns	ns	ns	ns	-*	ns
	GO6	ns	ns	+	+	ns	+	ns	ns
	LA5	ns	ns	ns	ns	ns	ns	ns	ns
	AL0	ns	ns	ns	ns	ns	ns	ns	ns
	P16	ns	ns	ns	ns	ns	ns	ns	ns
	SE2	ns	ns	ns	ns	ns	ns	ns	ns
	SE5	ns	ns	ns	ns	ns	ns	-*	ns

DISCUSSION

The present chapter aimed at exploring the long-term fitness effects of MIT on several clones of *D. pulex* submitted to two main conditions: cumulative multigenerational exposure or ancestral-only exposure to two putative sub-lethal concentrations of the biocide. Overall, a fitness alteration was observed after three successive generations of exposure to the low concentration, though depending on the clone, fitness returned to control levels following 3 generations of high concentration exposure. By contrast, ancestral exposure (P and F1) did not lead to measurable negative impacts on fitness in F3, suggesting that MIT does not trigger transgenerational effects in *D. pulex*. Clone heterogeneity was observed in the response of various life-history traits and by extension in fitness. In the following, we propose hypotheses to explain the observed patterns, and discuss them in the light of the literature.

Transgenerational vs multigenerational effects

Following an exposure to MIT at low or high concentrations (LM, HM) from P to F1 generation, the overall survival, growth, and reproductive performances of their F2 and F3 offspring not directly exposed to MIT were not different from the controls. Therefore, our design did not reveal a general pattern of transgenerational effects *per se*, at either LM or HM. Nonetheless, one particular lineage (P16) showed a transient reduction in fitness after parental exposure to HM, with a drastic drop in fitness from F1 to F2, followed with a significant recovery in F3. This suggests the possibility of some delayed effect mediated via damage incurred through F2 exposure at the germ cell stage (Figure 2-1). However, the fact that only this lineage exhibited this transient pattern does not lend support to the hypothesis of a general mechanism of MIT toxicity specifically affecting the germline. MIT has been shown to affect development in zebrafish through a miRNA-mediated disruption of the thyroid endocrine system (S. Lee et al., 2022), yet we are not aware of any published effects of MIT to the germline itself.

More generally, the non-observation of transgenerational effects on the studied life history traits, and thus on fitness, may well indicate that MIT does not induce such effects in the model species. Alternatively (and perhaps more realistically), it may result from an inability of the experimental design to detect such effects, either due to the choice of concentrations and exposure conditions applied to the ancestors, or to the survey conditions in F3. Last, despite the high level of replication implemented per chemical condition ($n = 60$, i.e., 10 isolated representatives of 6 different lineages), the possibility of insufficient statistical power cannot be ruled out.

Comparatively, in the case of LM MG conditions, a negative impact of cumulative exposure was observed over 3 generations, implying a parental transmission of detrimental effects, as the fitness of daphnids globally tended to be diminished in F3 compared to previous generations (but see section on inter-clone variation). On the contrary, under HM MG conditions, 5 out of 6 lineages showed a progressive return to control-like levels from F1 to F3 - read as acclimation - which expressed more or less rapidly and completely according to lines. While we would have intuitively expected lower concentrations to have a much less negative impact than high concentrations, the dose-response relationship appears to be more often non-linear than linear, and in some cases non monotonous

(Calabrese, 2004). Beneficial effects at low concentrations, known as hormesis, are increasingly reported in ecotoxicology and thought to express the ability of organisms to cope with moderate stress through homeostatic mechanisms (Sebastiano et al., 2022). In the present study, though, the lower dose did not prompt a compensatory mechanism, which was also reported by Parisot et al. (2015) in the case of DNA alterations in *D. magna* after exposure to gamma radiation for 3 generations. However, it is also possible that in the case of MIT, repair mechanisms are only triggered above a threshold of damage, and the activation of defense mechanisms could only be a matter of gap in dose or time (by accumulation of damages over generations). For example, in *Caenorhabditis elegans*, the continuous exposure over more than 10 generations to ionic liquids induced alternately stimulation and inhibition of reproductive output under both high and low concentrations (Yue et al., 2021; Shi et al., 2021). In the present instance, without knowing responses over more than the F3 generation, we can only observe the convergence of LM and HM multigenerational responses.

Interestingly, while we failed to detect transgenerational impacts under ancestral exposure only conditions, the observed improvement in fitness after three generations of chronic exposure to high concentrations (HM MG) are indicative of positive inter- or transgenerational effects, opening the question of their adaptive value. In theory, non-genetic adaptive transgenerational effects are expected under changing environments, and when parents can anticipate the environment of their offspring (Herman & Sultan, 2011). Such effects can be mediated by the transmission of epigenetic, cytoplasmic, somatic, nutritional, environmental, and behavioral variation (Bonduriansky & Day, 2009). In the present case, we are unable to identify the beneficial factors transmitted to the offspring, as it might be possible that daphnids ate more in the presence of MIT (nutritional factor), that they allocated more energy to the offspring as compared to control conditions, or that MIT had some epigenetic impact passing the generational barrier⁵².

⁵² Next chapter will try to get more insights into these potential mechanisms.

Lastly, it should be noted that in our search for hypothetical phenotypic and fitness changes exclusively induced by ancestral exposure to MIT, we designed the experiment in such a way that the focal generation F3 was reared under control conditions only. Yet, it might be possible that if subsequently challenged with MIT, F3 TG individuals would have performed better than control individuals, i.e. transgenerational effects could be expressed under an MIT polluted environment rather than in a benign environment. Indeed, the direction of transgenerational effects has been shown to depend upon the environment experienced by the offspring (Guillaume et al., 2016). In our experiment, this hypothesis is supported by the behavior of MG F3 individuals, although we could not distinguish the specific consequences of direct vs indirect exposure, which cumulated in MG F3. Additional support for this hypothesis may be seen in a recent meta-analysis covering a large range of taxa showing that, especially in annual plants and invertebrates, the timing of ancestral exposure influences the outcome in terms of transgenerational effects. Such effects were strongest when ancestors were exposed at early stages, and concern mostly juvenile traits in the offspring, while they tend to decrease as offspring mature (Yin et al., 2019).

Delayed effects of sublethal concentration considered safe

The delayed effects of LM continuous exposure on daphnids fitness are of concern as they reflect an underestimation of MIT toxicity (Kresmann et al., 2018). The MG.08 type exposure was considered as the most realistic scenario (compared to the others), as freshwater bodies of Northern Europe receive rainfall events with such frequency that even biocides with short degradation half-lives are still likely persistent in these environments (Bollmann et al., 2017). The initial LM effective concentration measured in the present chapter was 0.28 (0.06) $\mu\text{g/L}$ (mean and sd, Table S2-4), which is below measured effluent concentrations from wastewater treatment plants (from 0.350 to 0.860 $\mu\text{g/L}$), but above those in rain and storm waters (maximum 0.11 and 0.15 $\mu\text{g/L}$ respectively), following measures in Paijens et al. (2020). From initial effective concentrations, we predicted a 50% degradation over 7 days in tap water, which is in accordance with Rafoth et al. (2007). In that paper, the authors also reported MIT degradation in river water with 100% MIT degraded at 7 days at 23°C (and 30% at 7 days in 4°C water), which seems long enough to suppose MIT to be relatively persistent given the regular and multiple discharges in the environment.

Under such low concentrations, we nevertheless observed an increase of mortality during the course of 3 generations of daphnia (with a generation time of 10-11 days here), which standard toxicity tests could not show. Indeed, the no observed effect concentration estimated for *D. magna* by manufacturers cannot be considered as sublethal concentrations (21-day NOEC = 0.0442 – 0.183 mg/L,

ECHA, 2015). Moreover, the detrimental effects of MIT in general were determined here by examining the bimodal distribution of individual fitness, while the comparison of other endpoints alone would have missed this information. Together, these results call for evolutionary approaches in ecotoxicity testing, as more adapted to the actual goal and purpose of ecotoxicology (Straub et al., 2020).

Intraspecific variability

For all endpoints, lineages displayed uneven responses, consistently with acute toxicity results, not only in their dose-responses but also in their time-response. It seems that as the number of exposed generations cumulate, fitness divergence intensifies among clones, reflecting differences in genetic composition. The divergent patterns noticeable in F3 were not visible in the F1 generation, and thus, again, could not be detected under standardized tests. See chapter 1 for additional comments on evolutionary toxicology and benefits of including intraspecific variability in ERA.

CONCLUSION

The objective of this chapter was to measure latent effects of mild MIT concentrations to *D. pulex* in various exposure setups. First, we found lethal and sub-lethal effects that changed over generations: these were evident in the case of direct exposure, but not in the case of parental or grandparental exposure only. Then, we showed that continuous exposure to environmental MIT dose induces non-negligible adverse effects on a non-target species. Lastly, these results reflected the importance of intraspecific variability in toxicity assessment since the intensity and/or temporality of all the effects described above were line dependent.

From the phenotypic to the molecular scale

Chapter 2 shows the responses in terms of fitness-related life-history traits in individuals from day 5 to day 21 of life, and mainly highlights the increased mortality of *Daphnia* exposed to MIT. For females that survived to day 21, it is difficult to identify a trait that is clearly altered by biocide exposure, and responses appear to be also particularly variable between clonal lineages. However, an evolution of responses over generations was observed, between F1 and F3, in the case of continuously exposed animals, strongly suggesting multigenerational effects and thus an effect on reproductive females. To gain further insight into the impact of MIT, we then conducted a study of transcriptomic responses in females reared simultaneously and under similar conditions to the females in Chapter 2, focusing on individuals that survived 20 days of testing. The results of this study are presented in Chapter 3.

CHAPTER 3 - Insights into the molecular mechanisms of methylisothiazolinone toxicity: transcriptomic modifications in the water flea

ABSTRACT

The integration of omics in environmental risk assessment (ERA) opens the way to a deeper understanding of underlying mechanisms responsible for the toxicity of emerging pollutants. The toxicity mechanisms of the emerging pollutant methylisothiazolinone (MIT) have been rarely evaluated on invertebrate non-target species despite their central position in ecosystems. In this chapter, we aimed to conduct a preliminary screening of potential molecular functions impacted by the exposure to this biocide in the key freshwater species *Daphnia pulex*. MIT produces free radicals and destroys protein thiols, leading to cell damage and death, and it can also cause metabolic toxicity, developmental disturbance, and epigenetic deregulation, among other effects. We showed here that these effects were under after various environmental contaminant treatments, including exposure to low concentrations (0.8 µg/L), as well as multigenerational and transgenerational exposure in six lineages of daphnids. All exposure conditions induced transcriptomic modifications, with enriched GO terms related to catabolic processes, proteolysis, endopeptidase activity, mitosis processes, amino-acid synthesis processes, extracellular organization, chemical homeostasis/ion transport, apoptosis, hormonal regulation, developmental processes, and antioxidant activity. Moreover, epigenetic regulation seemed to have a key role in the response to MIT toxicity that was shared among all lineages. However, no pattern linking individual fitness responses reported in the second chapter and enriched GO terms was found.

INTRODUCTION

Methylisothiazolinone (MIT) is a broad-spectrum biocide primarily used as a preservative in personal care and household products, in concentrations assumed to be innocuous to ecosystems (ECHA, 2014). However, EU biocidal product regulation does not cover all of the different applications of this biocide (Wieck et al., 2016), and data on actual concentrations of MIT in surface waters are too limited to allow reliable estimations of the ecological risk associated with this biocide (Paijens, Frère, et al., 2020; Nowak et al., 2020). Therefore, further risk assessment and evaluation of the impacts of this preservative on exposed populations and ecosystems are needed due to potentially underestimated risks posed by MIT to aquatic ecosystems. (Kresmann et al., 2018).

In the second chapter of this thesis, we demonstrated a clear negative impact of MIT on *Daphnia pulex* fitness across six clonal lineages. We propose here to identify potential underlying molecular mechanisms of these phenotypic outcomes through the analysis of gene expression at the transcriptional level.

The toxicity of MIT stems from the production of free radicals and the destruction of protein thiols, inhibiting respiration and ATP synthesis, which quickly leads to cell damage and death (Williams, 2007). Therefore, we expect that the mechanisms of response to MIT exposure would be linked to the antioxidant system, and in particular glutathione (GSH). Indeed, bacterial growth inhibitory activity of isothiazolinones was extinguished by the addition of thiol-containing materials such as GSH (Collier, Ramsey, Austin, et al., 1990). Moreover, in humans, allergic contact dermatitis caused by isothiazolinones was inhibited by the application of a GSH-enriched emollient (Gruvberger & Bruze, 1998; Isaksson, 2015).

To our knowledge, the only study that relates the molecular basis of the effects following MIT exposure alone in non-mammal or bacterial species concerns developmental disturbance in zebrafish embryos (S. Lee et al., 2022). The authors examined the transcriptional profiles of genes related to the hypothalamus-pituitary-thyroid in larvae exposed to MIT and octylisothiazolinone (OIT) in parallel with T3 and T4 levels. The particular upregulation of two genes (dre-miR-193b and dre-miR-499), after exposure to 300 µg/L of MIT or 30 µg/L of OIT, suggests epigenetic deregulation linked to thyroid endocrine system disruption. In conjunction with chloro-methylisothiazolinone (CMIT), early life exposure to MIT induced morphological changes (tail and body bending), cardiac developmental issues, and affected locomotion behaviour (Chatterjee et al., 2021). These adverse effects happened simultaneously with global DNA hypermethylation, and proteomics profiling revealed potential mechanisms explaining cardiac failures. In invertebrates, exposure to CMIT/MIT can also exert metabolic toxicity. In *Caenorhabditis elegans*, Y. Kim & Choi (2019) showed that early-life exposure

was particularly linked with O-linked N-acetylglucosamine transferase (OGT) pathway and subsequent nutrient metabolism leading to health damages. In mammals, Bauer et al. (2015) showed that OGT is involved in DNA demethylation via TET protein regulation. In another model species, *D. magna*, CMIT/MIT exposure provoked adverse effects on reproduction that were potentially linked to a change in regulation of the storage protein vitellin (J. Kim & Choi, 2022, preprint). In rat and human cell lines, CMIT/MIT impaired mitochondrial function and dynamics (oxidative stress), resulting in endothelial barrier dysfunction, associated with systemic health effects (D. Kim et al., 2022). Park & Seong (2020) identified MIT-induced apoptotic cell death via oxidative stress, and impaired structure and function in human bronchial epithelial cells.

Altogether, the mechanisms of toxicity of isothiazolinone biocides involve inhibition of metabolic enzymes, disruption of protein thiol groups, free radical generation, oxidative stress, apoptosis and necrosis (Ettorre et al., 2003; Di Stefano et al., 2006; Frosali et al., 2009).

Based on results presented in the second chapter, this study aimed to investigate gene expression patterns in response to MIT exposure, with the goal of providing some mechanistic support to the phenotypic patterns observed, or revealing additional underlying sub-lethal responses we did not uncover previously. Two concentrations of MIT (0.8 and 50 µg/L) were applied to six clonal lineages of *D. pulex* that were exposed both directly and indirectly over four successive generations. RNA-Seq analysis was conducted to track transcriptomic changes and their timing during (1) acclimation to high MIT concentration, (2) delayed response to low MIT concentration. We also compared such changes across lineages and searched for transgenerational patterns at this molecular level.

MATERIAL AND METHODS

Experimental design

Daphnids from six genetically-distinct lineages were reared over four generations in groups of 25 animals in 1L glass jars (these also served as broodstock as described in chapter 2). Daphnids were reared in dechlorinated tap water with a 16:8 L:D photoperiod and fed every day *ad libitum* with a mixture of *Chlorella vulgaris* and *Desmodesmus subspicatus*. Alongside controls, 21 day-exposure to MIT followed two schemes (Figure 3-1): either continuous exposure from generation P to F3 (MG), or ancestral exposure from P to F1 (TG). In the latter, the exposure to contaminated water stopped at the 12th day, with transfer of daphnids to uncontaminated water, to ensure that offspring born on the 17th day (or later) would have been directly exposed only at the germ cell stage (F2). Two concentrations of MIT were tested: high concentration 50 µg/L (HM), and low concentration 0.8 µg/L (LM). At least three

jars were maintained per condition (Line X Scheme X Dose X Generation). At the end of the 21 days, animals from MG-F1, MG-F2, MG-F3, TG-F2, and TG-F3 conditions were flash frozen in liquid nitrogen, lyophilised over 48h, and kept at -80°C before RNA extraction.

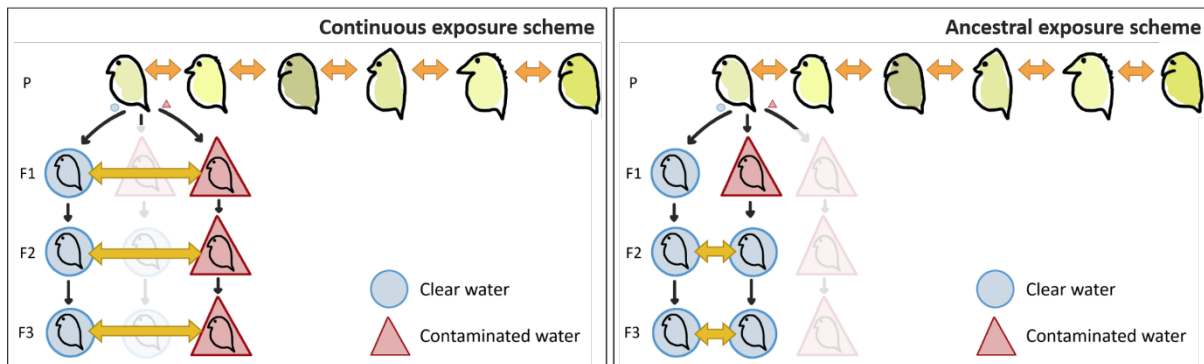


Figure 3- 1 Identification of contrasts performed on multigenerational and transgenerational treatments. Arrows denote the different statistical comparisons that were carried out to identify differentially expressed genes among the six clonal lineages and exposure schemes.

Extraction and sequencing

RNA extractions were performed using a NucleoSpin RNA Mini kit for RNA purification (Machery-Nagel; ref. 740955), following manufacturer's instructions with some modifications. To ensure rapid sample processing, only 12 samples were processed at a time. For each sample, approximately 10 to 30 lyophilised clones were transferred to an MN Type D bead tube (Machery-Nagel; ref. 740814), containing a 3mm tungsten steel bead and 354µL lysis buffer (100:1 ration of buffer RA1:β-mercaptoethanol). Samples were agitated on a Qiagen bead mill for 2min at 30Hz. Lysates were transferred to 1.5mL sterile, nuclease-free tubes and incubated overnight (ca. 12h) at 4°C. Subsequently, lysates were passed through the first filter by centrifugation at 11×g for 1min at 4°C. The resultant elutes were kept on ice, and each carefully mixed with 350µL of cold (ca. -5 to -10°C) 70% ETOH via repeated aspiration and ejection. This mix was passed through the subsequent NucleoSpin filters, centrifuged for 30s at 11×g and 4°C, with the resultant elutes discarded. Filters were each rinsed with 350µL of buffer MDB centrifuged for 1min at 11×g and 4°C. Filters were transferred to clean tubes, and incubated with 95µL of DNase for 15min at room temperature. Following DNase treatment, each filter was washed with subsequent buffers (RAW2 and RA3) and centrifugation series as outlined in the manufacturer's protocol. Empty filters were given a final 20s 11×g centrifugation to remove trace/residual ETOH. Filters were then transferred to labelled, sterile nuclease-free tubes, and left uncapped for 5min to allow for drying and evaporation of surface ETOH. Subsequently, 60µL of nuclease-free water was added to each filter, followed by 5min of incubation at room temperature, then centrifugation at 11×g for 1min. Elute was collected from each tube and re-

deposited on their respective filters, followed by 5min of incubation at room temperature and centrifugation at 11×g for 1min. A 10µL aliquot was reserved for each extraction, with the remaining 50µL deposited into a well of a 96 well plate for subsequent pre-sequencing treatment and labelling. Aliquots were used to determine sample quality and RNA concentration. Quality was assessed via absorption ratios using a DeNovix spectrophotometer. RNA concentrations were determined via colorimetry using Qubit RNA quantification kits. RNAseq was performed at the GeT-PlaGe core facility, INRAe Toulouse. RNA-seq libraries were prepared according to Illumina's protocols using the Illumina TruSeq Stranded mRNA sample prep kit to analyze mRNA. Briefly, mRNA were selected using poly-T beads. RNA was fragmented to generate double stranded cDNA, and adaptators were ligated to be sequenced. 11 cycles of PCR were applied to amplify libraries. Library quality was assessed using a Fragment Analyser and libraries were quantified by QPCR using the Kapa Library Quantification Kit. RNA sequencing was performed on an Illumina NovaSeq 6000 using a paired-end read length of 2x150 pb with the Illumina NovaSeq 6000 sequencing kits. For both DNA and RNA sequencing, quality control was performed with fastQC v0.11.2, and contamination search executed with bwa v0.7.15-r1140 against Ecoli FRIK2069, nCoV-2019, phi and yeast.

Transcriptome assembly

Raw reads were assembled separately for each lineage (37-40 libraries per lineage) using Trinity-v2.11.0 (Grabherr et al., 2011), with the genome of *D.pulex* KAP4 line (Bioproject PRJNA777597). The choice of reference genome was made from the results of the alignments of DNA reads of each lineage, conducted on multiple reference genomes (FastQ Screen v0.14.0, Wingett & Andrews, 2018). First alignments of reads on reference genomes were performed with Bowtie 2 version 2.3.4.3 (Langmead & Salzberg, 2012). Transcriptome reconstruction on RNA-seq with genome-guided *de novo* assembly, was run with the following options: --min_kmer_cov 2 (corresponds to the minimum count for k-mer to be assembled during the Inchworm phase, default=1, the assembly is still of high quality and requires less RAM, but lowly expressed transcripts may be more fragmented); --jaccard_clip (minimize falsely fused transcripts); --genome_guided_max_intron 500; transcript quantification with RSEM. Genome-guided *de novo* is a method whereby reads are first aligned to a reference genome, partitioned according to locus, followed by *de novo* transcriptome assembly at each locus. This enabled the capture of sequence variations contained in RNA-Seq samples. The completeness of transcriptome assemblies was assessed according to conserved ortholog content with BUSCO (v5.3.2) and the reference database "arthropoda_odb10", and read representation of the assembly with Bowtie2.

Differential Analysis

Expression (number of read copies) was determined at the gene level, i.e., grouping alternative isoforms of a same gene. Differential expression was analysed with DESeq2 (v1.38.3). Each dataset (n = 6) was reduced such as to avoid spurious effects of very low frequency transcripts on the global variance estimation and further library normalization. To do so, we filtered out genes for which there were less than 3 samples with counts greater than or equal to 10. The statistical model was based on a global factor encompassing all 13 experimental conditions per lineage, i.e., ControlF1, ControlF2, ControlF3, MG08F1, MG08F2, MGO8F3, MG50F1, MG50F2, MG50F3, TG08F2, TGO8F3, TG50F2, TG50F3 (n = 13, number of replicates in Table S3-1). Contrasts were performed in order to compare each exposure condition to its corresponding control (e.g., F2_MG08 vs F2_Control).

Functional annotation and Gene Ontology enrichment test

Differentially expressed genes (DEGs) were annotated via BLASTx against the *D. pulex* proteome retrieved from UniProt (328993 proteomic sequences, as retrieved from UniProt in April 2023). Functional annotation of the proteome was carried out with Gene Ontology, using the categories Biological Process, Cellular Component, and Molecular Function (see Supplementary Table S3-6), and further used to test for enrichment analysis using BiNGO (Maere et al., 2005), as implemented in Cytoscape. To do so, we used a hypergeometric statistical test followed by a Benjamini Hochberg False Discovery Rate correction with $P = 0.05$. Additionally, DEGs were BLAST searched against the KEGG database. In cases where DEGs were annotated as “uncharacterized proteins”, we searched for conserved domains using InterProScan, and if unsuccessful, we went back to original transcripts and checked if these were non-coding RNAs, using BLASTn.

RESULTS and DISCUSSION

Quality of transcriptomes

The BUSCO results show that more than 90% of the 1013 orthologous genes are found in 5 of the 6 transcriptomes, indicating that they contain biological information expected for arthropods (Table S3-2). The low percentage of retrieved orthologs for the SE5 transcriptome (72.2%) might alert to a lower quality than in other lineages, while the SE2 transcriptome score was the highest (98.5%). A large majority of complete genes are found as duplicates (over 70% in 5 out of 6 lineages), which was expected as we did not filter the numerous isoforms. The overall alignment rate of reads on

transcriptome varies from 75.5% (GO6) to 83.8% (SE2), which was deemed an acceptable quality standard (Table S3-3).

Differential Expression Profiles

All exposure conditions induced transcriptomic modifications. Quantitatively, the number of DEGs (Table S3-5) did not exhibit any trend consistent with concentration (0.8 vs 50 $\mu\text{g/L}$), mode of exposure (MG vs TG), or generation (F1 to F3, F2 to F3). We only found a pattern of decrease in DEG number between F1 and F3 under HM concentration (MG50) in 4 out of 6 lineages (AL0, GO6, LA0 and SE5). This pattern might be consistent with the acclimation hypothesis underlying phenotypic and fitness changes (chapter 2). However, this hypothesis would not hold for SE2, in which the number of DEGs increased slightly in F3. The P16 MG50 F3 condition was not considered reliable as it encompasses a single replicate (Table S3-1).

Qualitatively, the number of DEGs shared across conditions was assessed within each lineage and for each mode of exposure (TG or MG), as illustrated on Figures 3-2 and 3-3. Under TG (Figure 3-2), the number of DEGs found common to all conditions (generation and concentration) in each line varies from 8 (AL0) to 61 (SE5), whereas the largest groups of DEGs are found under unique conditions (except in GO6, where TG08 et TG50 share 472 DEGs in F3). Under MG, the 6 studied conditions are illustrated on Venn diagrams for each lineage separately (Figure 3-3). While the number of DEGs shared across all conditions varies from 8 (LA0) to 21 (SE2), the largest groups of DEGs appear again as exclusive to a single condition. Moreover, this condition is not the same across lines: F1-MG08 (SE2 and AL0), F1-MG50 (SE5, LA0, GO6), and F3-MG50 (P16) (yet this last one should be interpreted with caution, as already mentioned). Excluding P16, it appears that the highest number of DEGs is always observed in F1, i.e., at first MIT encounter. When comparing lineages under a given condition, the first common feature is that very few DEGs are shared (from zero to 8 DEG shared by all 6 lines, and also very few shared by 5 lines). As under TG, the largest DEG sets are usually found as exclusive to a given lineage (SE2, AL0 and P16, under condition F1-MG08).

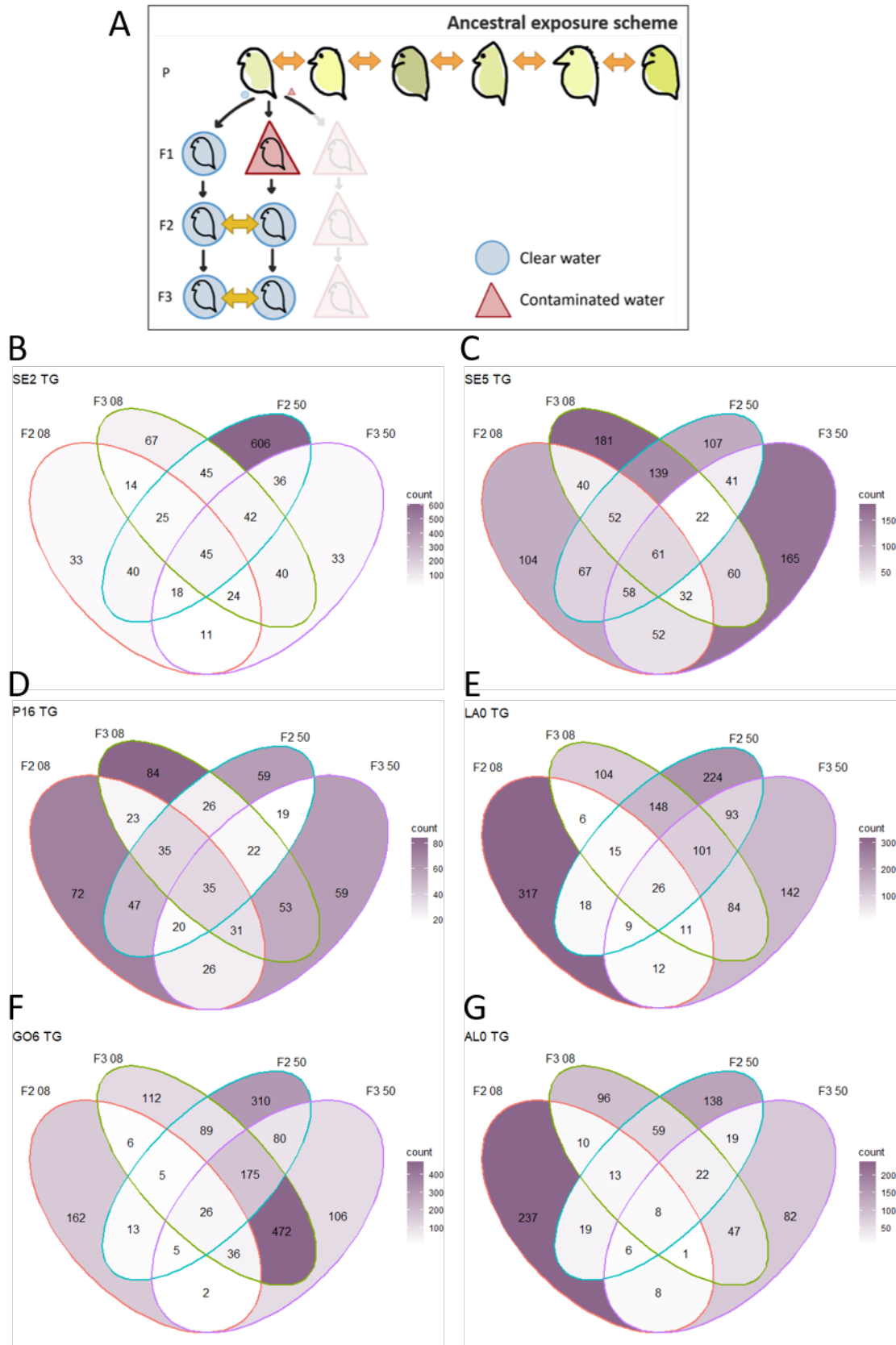


Figure 3- 2 Number of differentially expressed genes (DEG) for each lineage in the ancestral exposure scheme (TG, P and F1-only exposure), maternal and germ cell exposure (F2) or transgenerational exposure (F3), under high (50) or low (08) MIT concentration.

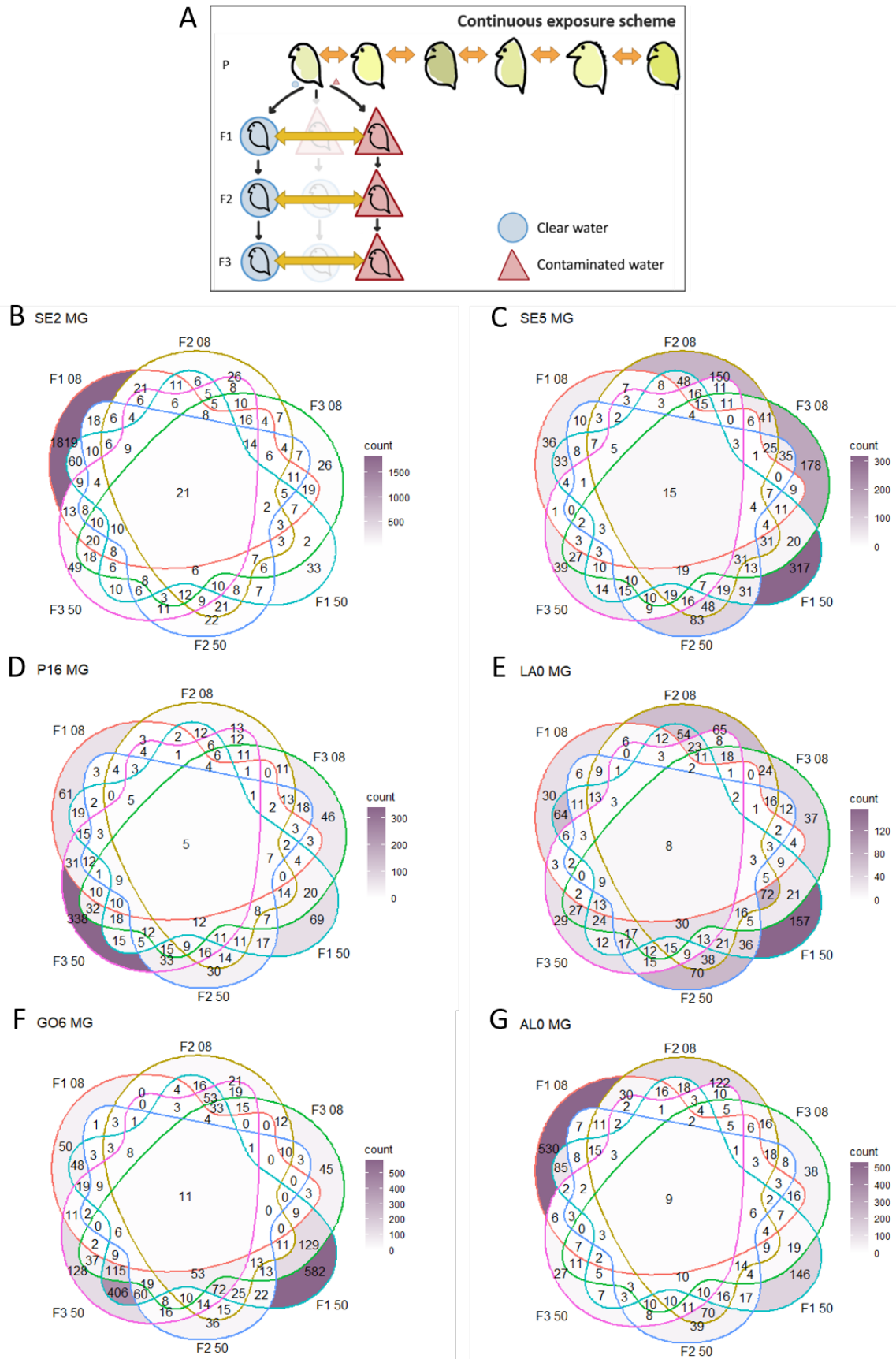


Figure 3- 3 Number of differentially expressed genes (DEG) for each lineage in the continuous exposure scheme (MG), from F1 to F3, under high (50) or low (08) MIT concentration.

Functional annotation

In all lineages, at least 73% of DEGs were successfully annotated against the *D.pulex* reference proteome (Table S3-6), with the highest score held by SE2 DEGs (88%) which was probably due to its higher quality. Among these annotated DEGs, approximately half corresponded to at least 1 GO term (46-57%), and less than a third corresponded to at least 1 KEGG term (21-36%), except for AL0 (75%). Within each treatment, 26 DEGs were shared by at least 5 lineages (Table 3-1). Twenty-five of these DEGs were annotated as “uncharacterized” (protein or locus) and actually corresponded to non-coding RNAs (ncRNA). These ncRNAs were between 200 and 1600 nt long, which put them in the “long non coding” (lnc)RNA category (Mattick et al., 2023). The last DEG (in TG08 F2) matched with 93% identity with “C1q and tumor necrosis factor-related protein-like protein 2 [*Daphnia pulex*]”.

Table 3- 1 Differentially expressed genes (DEGs) shared between at least 5 lineages in the different treatments. Every gene corresponds to ncRNA, except for E9GCS8.

Uniprot ID	F1		F2				F3			
	MG		MG		TG		MG		TG	
	08	50	08	50	08	50	08	50	08	50
E9H640		X	X	X	X			X	X	X
E9GL04		X	X	X	X		X	X		X
E9I515			X	X	X	X	X	X	X	
E9GNC6	X	X			X		X			X
E9GDY4			X	X		X				X
E9GJB0						X	X		X	
E9GAZ6			X				X			
E9G2I9	X				X					
E9FV08		X			X					
E9H348							X			X
E9FSW5								X		X
E9G6A9				X			X			
E9GDC7						X	X			
E9GFS3				X				X		
E9GIR6	X									
E9H585	X									
E9G352		X								
E9GDW6		X								
E9GSC7		X								
E9HCW3		X								
E9HZX9										X
E9GP08							X			
E9I1V1							X			
E9FT06					X					
<i>E9GCS8</i>					X					
E9I0B1					X					

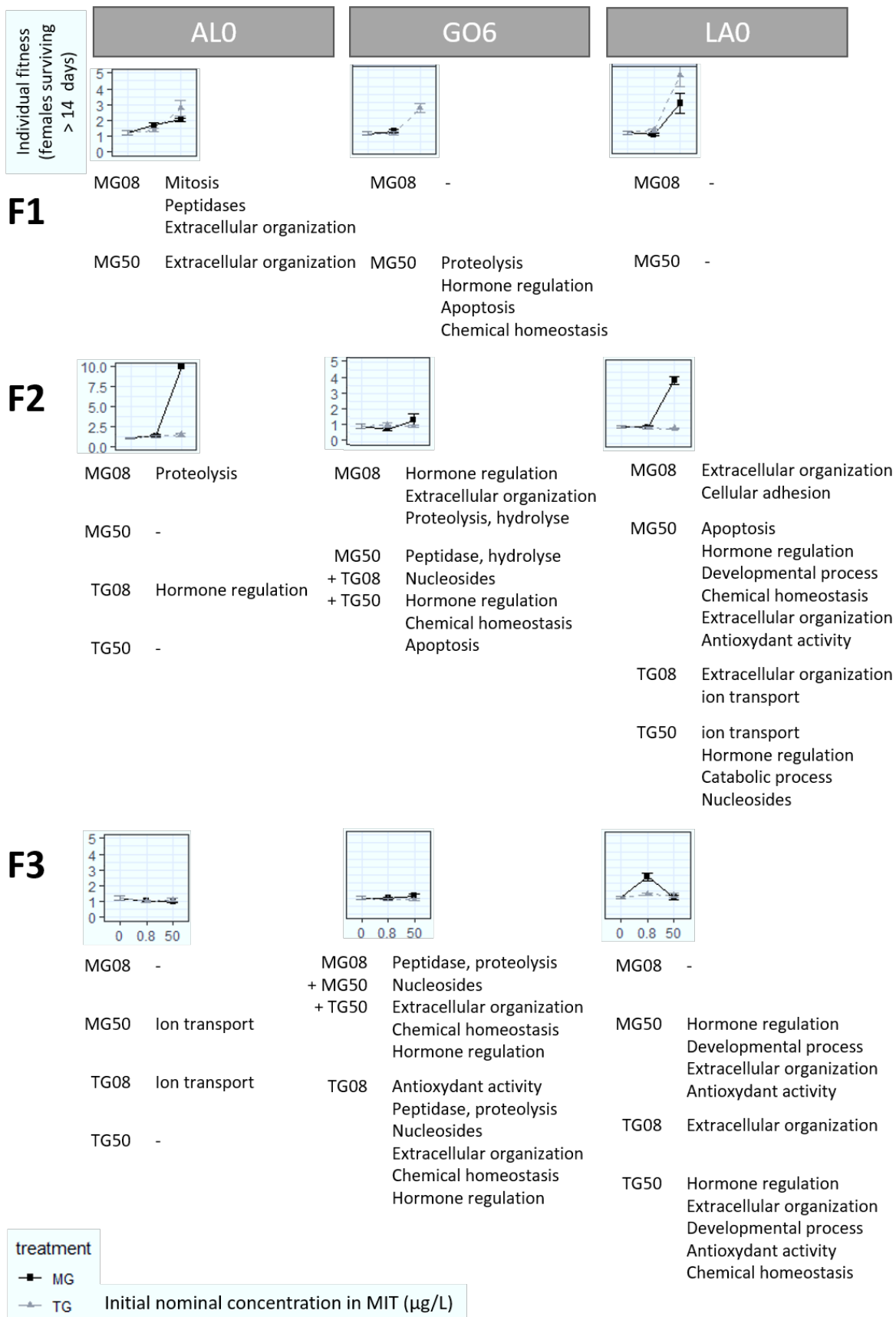


Figure 3- 4a Summary of over-represented GO terms identified among the transcripts of significantly up- and down-regulated genes in MIT exposure setups compared to controls (enrichment tested with BiNGO). (1/2)

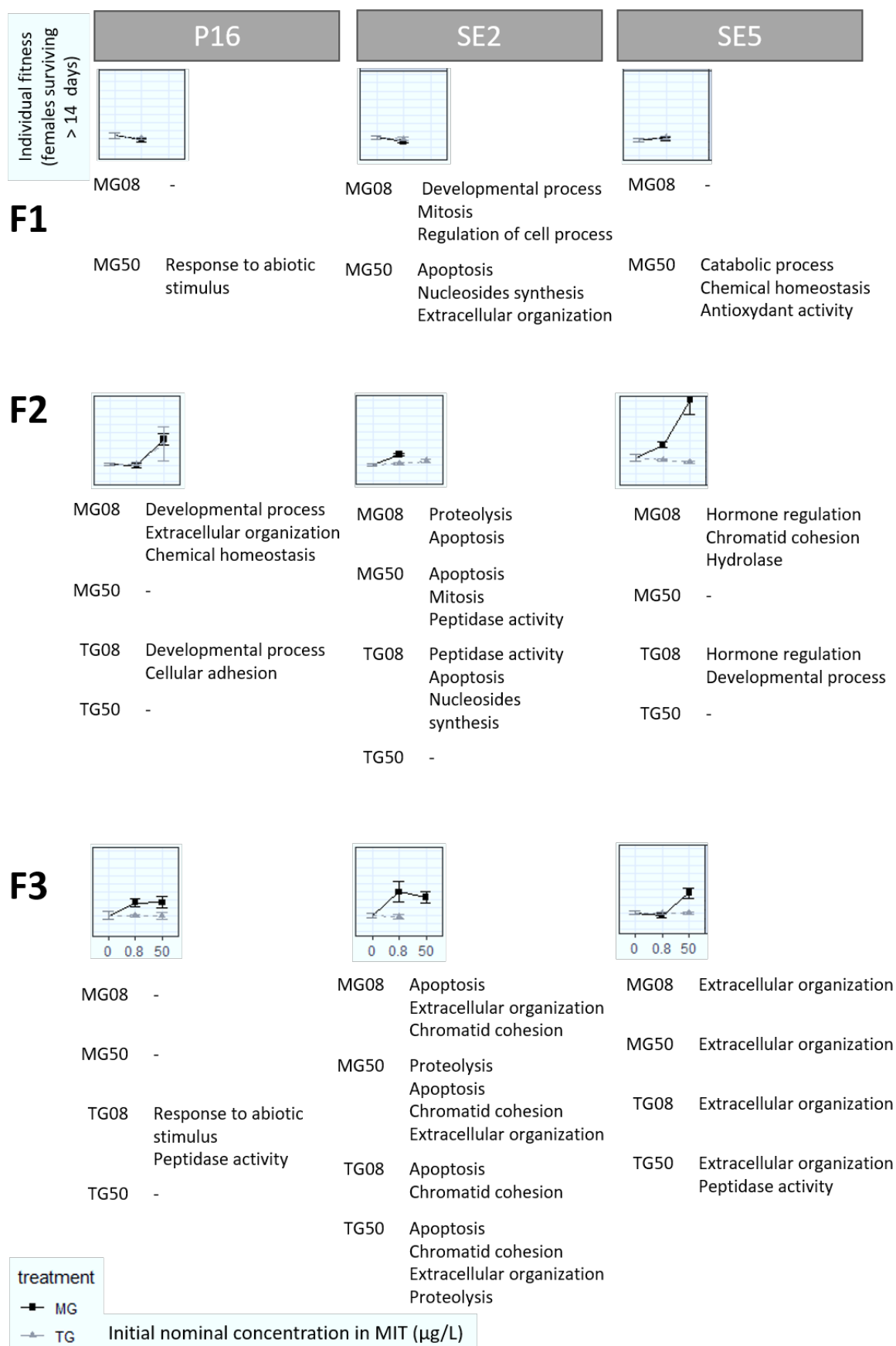


Figure 3- 4b Summary of over-represented GO terms identified among the transcripts of significantly up- and down-regulated genes in MIT exposure setups compared to controls (enrichment tested with BiNGO). (2/2)

The most common GO terms identified among the DEGs in MIT exposure setups compared to controls were catabolic processes, proteolysis, endopeptidase activity, mitosis processes, amino-acid synthesis processes, extracellular organization, chemical homeostasis/ion transport, apoptosis, hormonal regulation, developmental processes and antioxidant activity (Table S3-6). A synthetic view of enriched GO terms is displayed for the different treatments and lineages in Figure 3-4ab, alongside fitness responses of females surviving more than 14 days (chapter 2). Like for the quantitative comparison of DEGs, we could not find a pattern linking individual fitness response and occurrence of enriched GO terms. Some conditions under which individual daphnia fitness was impacted could be linked to the absence of enriched GO terms, e.g. AL0 F2 MG50, or the contrary, e.g. LA0 F2 MG50. Equally, some conditions could combine unchanged fitness and multiple enriched GO terms, e.g. GO6 F3 TG08, or none, e.g. SE5 F1 MG08.

Altogether we did not find a clear link between transcriptome activity and the delayed toxicity nor acclimation processes observed in chapter 2. Because transcriptome assembly was performed separately between lineages (due to resource limitations), we might have lost some power for intraspecific comparison. Moreover, only half of the DEGs were annotated against the *D.pulex* proteome, which reduced the range of detectable effects at the functional level.

In order to avoid excess handling stress prior to cryogenic preservation, we could not physically extricate maturing juveniles from female brood pouches. As such, extracted tissues combined both mature females and their embryos (at different instar stages). Whilst this effect was common to all conditions including controls, we cannot be certain that in some replicates the embryos' developmental genes do not overshadow the transcriptional response to the biocide. Therefore, any conclusions related to development function regulation should be tempered.

Furthermore, multiple alignments of DNaseq data on different reference genomes of each lineage (Figure S3-1) revealed a global homogeneity between all lines matching slightly better with the *D. pulicaria* genome except for the GO6 reads that appeared to better resemble the *D. pulex* D84A genome. In all lineages, between 40 and 50% of mapped reads hit once on the multiple *D. pulex/pulicaria* genomes, and between 15 and 50% of mapped reads hit multiple times on them, as the *Daphnia* genome presents a high rate of gene duplication (Colbourne et al., 2011). For AL0, LA0, P16, SE5 and SE2, very few reads hit specifically with *D. pulicaria* SC-F1-1A (<2%) and *D. pulex* KAP4 (<1%); for GO6, reads mapped specifically on *D. pulex* D84A (2% only once and 2% multiple times). This suggests that GO6 may represent a more divergent lineage within this species complex from the others (with *D. pulex* and *pulicaria* as sister species), which could be another source of variability to

watch for. Because of the proximity of the species and of the similarity of responses to MIT of GO6 with other lineages, all lines were kept for the analyses.

Interpretive caveats notwithstanding, we showed that all MIT exposure schemes elicited a reaction at the transcriptome level compared to control conditions, including transgenerational exposure. As expected, these effects included abiotic stress responses such as antioxidant activity, well-known as a short-term acclimation to electrophilic xenobiotics (Sthijns et al., 2016). Hormonal function regulation was also affected by MIT exposure, supporting the observations of endocrine disruption processes in vertebrates (S. Lee et al., 2022, 2023). Interestingly, the few DEGs shared among the response of all lineages to all treatments were ncRNAs, which have roles in post-transcriptional regulation and epigenetic inheritance (Fallet et al., 2023). In particular, here lncRNAs are concerned while S. Lee et al. (2022) reported an effect of MIT on the development in zebrafish through a miRNA-mediated disruption of the thyroid endocrine system. However, both small and long ncRNA have been found to contribute to transgenerational epigenetic inheritance (Chen et al., 2016; Mattick et al., 2023).

GENERAL DISCUSSION

In the context of evolutionary ecotoxicology, the present thesis aimed to achieve multiple objectives through an empirical approach using *Daphnia pulex* as a model species. Specifically, we investigated how genetic variation influences the response to MIT (biocide), an emergent pollutant. To do so, we exposed daphnids to different exposure modes (acute vs chronic) and timings (from single to multigenerational cumulative exposure), monitoring fitness-related traits under direct and indirect (parental) exposure. Additionally, we examined transcriptomic expression under the same conditions to identify molecular-level effects and mechanisms potentially involved in observed life history patterns. The work presented in these three chapters (Figure x) contributes to the field of evolutionary ecotoxicology by highlighting the importance of considering intraspecific variability in toxicity assessments and the potential for long-term effects of xenobiotic exposure on non-target species.

Chapter 1 demonstrated the occurrence of intraspecific variation in *D. pulex* sensitivity to acute exposure to MIT alone or in mixture with CMIT, using 8 clones originating from different populations or strains. This result joins a long list of findings on intraspecific variation in toxicology across all categories of organisms (e.g., microorganisms: Colpaert et al., 2000; plants: Meyer et al., 2010; crustaceans: Maltby & Crane, 1994; Barata et al., 2000; Lopes et al., 2006; Vigneron et al., 2015; Major et al., 2018; molluscs: Salice & Roesijadi, 2002; Côte et al., 2015; fish: Oziolor et al., 2014; etc.). It is also consistent with the idea that, due to different environmental and evolutionary history having shaped their genetic diversity, different populations or strains are not expected to produce similar responses to toxicants (Brans et al., 2021; Almeida et al., 2021; Romero-Blanco & Alonso, 2022). We observed a wide range in 48h-LC50 values (from 0.68 to 2.84 mg/L for MIT, and from 0.1 to 0.37 mg/L for CMIT/MIT), indicating a high dependence of *D. pulex* tolerance on the genotype used. Therefore, in an ERA context for example, the choice of the strain has its own importance for data extrapolation.

Chapters 2 and 3 addressed chronic effects, including those incurred after multiple generations of exposure, and after parental-only exposure. The experimental design was based on acute toxicity results (chapter 1), concentrating on 2 MIT test doses (one consistent with reported environmental concentrations, and a higher one expected to trigger stronger responses), and was restricted to 6 of the 8 lines mentioned in chapter 1, chosen as the most representative of global variation detected under acute exposure. This repositioning was technically required to counterbalance the complexity of the experimental design (3 generations x 6 lines x 2 exposure schemes x 2 concentrations) and daily trait measurements.

Chapter 2 focussed on the phenotypic responses of individual daphnia, specifically modifications in life-history traits (i.e. survival, reproduction, and growth), which were synthesized under a measure of individual fitness. Overall, a fitness alteration was observed after 4 successive generations of exposure to the low concentration, though depending on the clone, whereas fitness returned to control levels following 4 successive generations of high concentration exposure (except for line SE5). By contrast, germ cell or ancestral-only exposure (P and F1) did not lead to measurable negative impacts on fitness in F3, suggesting that MIT does not trigger transgenerational effects (Maggio & Jenkins, 2021), at least under the dosage tested. Only the P16-TG50-F2 individuals appeared to be affected by germ cell stage exposure, which on its own, is insufficient to conclude broader intergenerational effects of MIT on *D.pulex*. Clone heterogeneity was also observed in the response of various life-history traits, and by extension in fitness. However, by comparing the 2 distinct dynamics under continuous exposure schemes, we found arguments in favour of the occurrence of inter- or transgenerational positive and negative effects (Guillaume et al., 2016), as reflected by the improvement (in HM) or decrease (in LM) of performances from MG-F1 to MG-F3 individuals.

Chapter 3 expanded the scope of potential effects to the transcriptome level, providing insights into the potential molecular mechanisms responsible for the toxicity of MIT in *D. pulex*. Contrary to precedent results, significant numbers of DEGs revealed that all types of exposure to MIT elicited a response from daphnia, including ancestral exposure under low MIT concentration, which confirmed that the “absence” of effects concluded in the second chapter was only due to the traits under consideration. Whilst the mechanistic relevance of omics in ecotoxicology is well recognized (X. Zhang et al., 2018), the discrepancy observed in the present study also points to physiological value of snapshots of transcriptomic changes (van Straalen & Feder, 2012). Insights into functional annotations of DEGs revealed that transcriptome modifications related to catabolic processes, proteolysis, endopeptidase activity, mitosis processes, amino-acid synthesis processes, extracellular organization, chemical homeostasis/ion transport, apoptosis, hormonal regulation, developmental processes, and antioxidant activity. This is concordant with the little existing data in terms of processes concerned by MIT toxicity (Y. Kim & Choi, 2019; E.-J. Park & Seong, 2020; Chatterjee et al., 2021; Lee et al., 2022). Moreover, by taking a closer look at DEGs shared among lineages and conditions, we identified 25 long non-coding RNAs. LncRNAs have also shown significant transcriptional correlation with response to pesticide exposure in other arthropods (Etebari et al., 2015; Valenzuela-Miranda et al., 2017).

The main limitation of the study overall are the nullified F3 controls (all lineages) and F3 SEN2.TG.50 daphnia phenotypic data. Because the scale of the experiment led to inevitable desynchronization of births of daphnia along generations, from the synchronized start of P-1 generation, a time gap between treatments and lineages subsisted within generations. In the end, the total experiment for F1 generation was run during 24 days, the F2 during 33 days and the F3 during 29 days (Figure S2-2). Therefore, during the last generation for example, the daphnia of the last group (SEN2.TG.50 F3) were born 8 days after the first groups. The last groups tested (F3 controls of all lineages and SEN2.TG.50) produced surprisingly only few offspring. Since it mainly concerned animals that were never exposed and related to a precise period of time, we narrowed down potential reasons for this uncontrolled drop of reproduction. Water parameters were monitored weekly and did not disclose any particular change during the last week of the experiment (Figure S2-3), nevertheless the daily temperature monitoring revealed abrupt variations at the end of the experiment (range = 16.25°C to 20.0°C; Figure S2-2). Due to these observations, and the well known impact of temperature on daphnia development and reproduction (Goss & Bunting, 1983; Vanvelk et al., 2021), we decided to remove reproduction data from SE2.TG.50.F3 and all 6 lineages' C.F3. However, a closer survey of water parameters could have highlighted other changes of conditions that we missed and could have had some impacts. Moreover, new contaminants or parasites may have entered the experiment system water unnoticed, which is not verifiable.

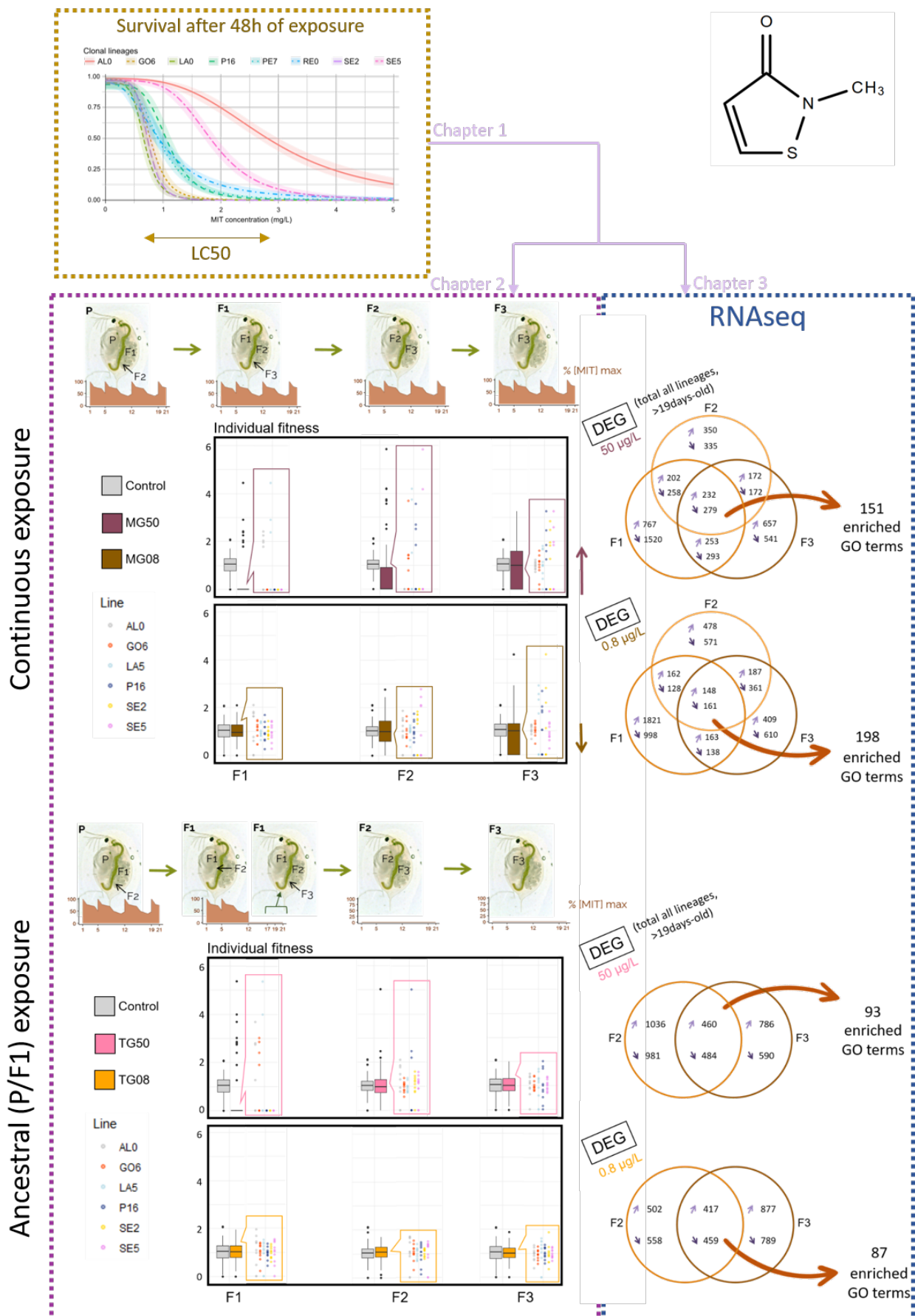


Figure x (continued). Acute toxicity was assessed on eight clonal lineages of *Daphnia pulex* species complex, leading to a broad range of LC50 values. Six of these lineages were selected and were included in a multigenerational exposure experiment conducted over four generations (from P to F3), and with either high (50 µg/L) or low (0.8 µg/L) nominal concentrations of MIT. Altogether, water fleas exposed to high doses of MIT from early developmental phase (F1) first experienced significant mortality and an important number of down-regulated genes in surviving females, followed by a quick recovery of individual fitness compared to controls in the case of offspring exposed only in the ovaries (TG50 F2) or indirectly (TG50 F3), paired with fewer DEGs. Females exposed continuously to high concentrations (MG50 F2 and F3) recovered over several generations, with various sensitivities among the lineages, and also with lower DEGs than in F1. Water fleas exposed to low concentrations of MIT did not show a change in individual fitness compared to controls in F1 (for any lineage); however, surviving females had the highest number of DEGs of all exposure schemes. While phenotypic responses of offspring reared in uncontaminated water remained similar to controls (TG08 F2 and F3), with reduced DEGs, the global fitness of continuously exposed daphnia decreased over generation (MG08 F2 and F3), with four out of six lineages experiencing increased mortality in either or both F2 and/or F3 generations (but with decreased number of DEGs).

Overall, 41 common GO terms were significantly enriched after exposure (when adding each lineage's results) which can be simplified into nine groups of functions/components: ribo/nucleotide binding, extracellular space, peptidase/hydrolase/proteolysis activity, ion homeostasis, macromolecule metabolic process, ATP binding, apoptosis, cohesin complex/meiotic process, and regulation of hormone levels/ecdyteroid/ketone biosynthetic process. MIT exposure also provoked a change of regulation of 26 genes for at least five out of six lineages under the various exposure schemes, 25 of them corresponding to lncRNAs.

With various intensity, every exposure to MIT that was tested here resulted in a waterflea response.

LC50 : Lethal concentration for 50% of the population, MG : 'Multigenerational'/continuous exposure, TG : 'Transgenerational'/ancestral exposure, 08 : maximal nominal MIT concentration of 0.8 µg/L, 50 : maximal nominal concentration of 50 µg/L, DEG : Differentially expressed genes, GO : Gene ontology, % [MIT] max : MIT concentration relative to maximal concentration (measured with LC-MS/MS).

1. Contributions for ecotoxicology

a. MIT concentration determination in water samples

Concentration determination of MIT and CMIT in water samples were conducted using liquid chromatography coupled to mass spectrometry, which is employed in the majority of studies focusing on the detection of micropollutants like isothiazolinone and related substances (Wick et al., 2010; Singer et al., 2010; Wieck et al., 2018; Silva et al., 2020; Paijens, Frère, et al., 2020). However, others have been using other more affordable but less sensitive techniques of spectroscopy like UV (also called diode array detector, DAD) in place of MS (Baranowska & Wojciechowska, 2013; Rosero-Moreano et al., 2014; Silva et al., 2020; S.-K. Park et al., 2020).

With the high concentrations used in chapter 1 (range 0.25-5 mg/L for MIT), the method was reliable and efficient. But with lower concentrations used in chapters 2 and 3 (maximum 50 or 0.8 µg/L), the quantification of MIT was difficult, and because the “08” (LM) samples concentrations were under the limit of quantification (LOQ), we assumed the degradation pattern of MIT over 7 days was the same as in “50” (HM) samples. Even if we were close to the LOQ, we are confident in the degradation rate we found for MIT as it was the same presented by Rafoth et al. (2007, under similar conditions, i.e. in tap water at 23°C). But while we are confident with respect to the relative concentrations, this is not the case for absolute concentrations. Therefore, even though the measured effective concentrations in the long-term experiment were about half those expected, the discoursed remained confined to nominal concentration.

Concerning the sensitivity and repeatability of the targeted measures of MIT, the triple quadrupole (LC/TQD) was noted to be more reliable than the Quadrupole time-of-flight (LC/Qtof) as expected (Chang et al., 2022); however, it still might not be sensitive enough to work with environmentally relevant concentrations. This aligns with instrumental limits of detection (LOD) and LOQ for MIT presented by Paijens et al. (2020), i.e. LOD_i of 0.25 µg/L and LOQ_i of 50 µg/L, but with final LOD of 5-13 ng/L and LOQ of 9.9-26 ng/L in urban and surface water by incorporating a solid-phase extraction (SPE) step in the workflow, which enriches the targeted micropollutants in the samples (Rafoth et al., 2007; Terzopoulou et al., 2015). Therefore, the introduction of an extra step such as SPE to increase the sensitivity in the determination of MIT in complex environmental waters seems essential for monitoring, as it is also essential in experimental conditions to study the effects of realistic doses of contaminants (often traces).

b. Toxicity modelling

For the modelling of survival after a short-term exposure (48h LC50), the GUTS-RED framework was applied (first chapter). This TKTD model is the most simple and powerful mechanistic approach available to our knowledge, and is recognized by EFSA as a ready-to-use tool for regulatory purposes (Ockleford et al., 2018; Forbes et al., 2021; Jager, 2021; Bart et al., 2021; Nickisch et al., 2022).

For the analysis of long-term phenotypic traits in the second chapter, we used an empirical statistical approach that describes, but does not explain, the mechanisms of responses to stressors. For example, the use of a mechanistic model applying Dynamic Energy Budgets (DEB) theory to toxicology ('DEBtox' or 'DEB-TKTD', Sherborne et al., 2022; Bart et al., 2023) could provide additional information and precision about specific parameters impacted by a given toxicant (e.g. assimilation, somatic maintenance, maturity maintenance). However, the lack of tested doses in our dataset precluded its use (only 2 doses tested against 5 doses minimum for reliable results of TKTD modelling), as the priority was set on the number of tested lineages instead of the number of doses. Alternatively, we compared life-history traits among treatments, and integrated them into an individual fitness index (from the Fisher model of individual reproductive value, 1999).

At the transcriptomic scale, we identified differentially regulated transcripts between MIT exposed daphnia and their untreated sisters, complemented with an enrichment analysis of GO terms, which helped to identify biological functions impacted by MIT contamination. Even with the unsolved reproduction failure of individualized unexposed daphnia in F3 (described above), we were able to detect common key functions affected by MIT exposure among lineages for each treatment type, which suggests a good level of confidence in our results. Nonetheless, other types of analyses could have been carried out after the identification of DEGs such as pathway analysis with KEGG annotations (Kanehisa & Goto, 2000), network analysis with correlation networks like WGCNA (B. Zhang & Horvath, 2005) or machine learning such as random forest models (Suppa et al., 2020; Choi et al., 2022) or artificial neural networks (Chapman et al., 2009). Whilst alternative analytical methods might have brought other insights from our data, the low functional annotation of our transcripts probably originate not only from the choice of a *de novo* assembly method for the transcriptome, but also from the large fraction of duplicated genes with unknown homology inherent to the *D. pulex* genome (Colbourne et al., 2011). Moreover, because the lineages were assembled independently from each other due to processing constraints, the transcripts that we failed to find annotations for were lost for inter-clonal comparison.

2. Contributions of experimental approaches in evolutionary ecotoxicology

a. Intraspecific diversity

Throughout the manuscript, “*Daphnia pulex*” was used to refer to the studied species, as determined morphologically, though it actually applies to a complex of species (Vergilino et al., 2011; Marková et al., 2013; Lynch et al., 2017; Maruki et al., 2022). Species complexes are groups of phylogenetically related species displaying little or no morphological differences (Hebert et al., 2004; Bickford et al., 2007; Elmer et al., 2010; Nava et al., 2014). The *D. pulex* complex can be subdivided into a small number of clusters maintaining biogeography and morphology characteristics, including lineages of *D. pulex*, *D. pulicaria*, and hybrids, from North America and Europe (Colbourne et al., 1998). These lineages are still undergoing a process of speciation via ecological isolation due to habitat preferences (ponds or lakes), with differences in hybridization occurrences, as wild populations of hybrids commonly found in North American populations in contrast to European populations that rarely show hybrids (Pfrender et al., 2000; Heier & Dudycha, 2009; Chin et al., 2019; Chin & Cristescu, 2021). In particular, wild hybrid lineages were found to be obligate apomicts, which was associated with polyploidy (Dufresne & Hebert, 1995, 1997; Vergilino et al., 2009; Pantel et al., 2011; Xu et al., 2015). Although the proximity of *D. pulex* and *D. pulicaria* has justified their consideration as a single taxon, because of their close phylogeny, their recent divergence time (approximately 2 Ma), and their hybrid viability and fertility, some variation observed in response to MIT exposure during our experiments may have originated from differences between species in addition to intraspecific influence. Indeed, in order to select the most appropriate reference genome for chapter 3 bioinformatics analyses, alignments of DNA reads of each line were conducted on multiple reference genomes which showed the presence of two species or hybrids. The results revealed a global homogeneity with all lines matching slightly better with the *D. pulicaria* genome except for the GO6 reads that appeared to better fit the *D. pulex* D84A genome (Figure S3-1). This particularity of GO6 clones could be the reason for discrepancies such as the subtle lower size of adults (Figure S2-26) and the absence of dead offspring all along the experiments (Figure S2-24) compared to other lines even in control conditions. While the presence of only one clone different from the others does not allow for a reliable interspecific comparison of the responses of *D. pulex* and *D. pulicaria*, the comparison of intra- and inter-specific response to stressors is essential to understand the relative importance of these two sources of variability – given a reasonable number of species and genotypes tested (Muyssen et al., 2005; Vanvelk et al., 2021; J. Kim et al., 2023).

Not only differences in toxicity response across and within species reflect both toxicokinetic and toxicodynamic variables (Baillie & Rettie, 2011), but intraspecific variability in quantitative traits is essential for a population to persist in the long-term, as it can determine if a population is able to

adapt to environmental changes (evolvability; Houle, 1992; Lande & Shannon, 1996). This was nicely illustrated by Loria et al. (2022) who found that diverse water flea populations did persist longer than a monoclonal population following copper exposure. On the other side, determining the origins of trait divergence is difficult. Common garden experiments can be used to decipher plastic responses from local adaptation to xenobiotics such as pesticides (Jansen et al., 2015; Brans et al., 2021; Almeida et al., 2021) or cyanotoxins (Schwarzenberger et al., 2021). For the experiments presented in this thesis, we picked the set of clonal lineages that would have the most diverging responses possible, thus we did not focus on particular populations, and therefore we cannot conclude on the relative tolerances of the populations at the origin of the lineages.

Due to the central position of daphnids in the food web, any change at population level have the potential to affect the function of their ecosystem. Unsurprisingly then, a growing number of papers are presenting toxicology and/or natural stress studies about *Daphnia* including intraspecific variability (e.g. Muyssen et al., 2010; availability of PUFA: Ilić et al., 2021; low temperature: Werner et al., 2021; oxidative stress and age: Ukhueduan et al., 2022; antipredatory morphological plasticity: Becker et al., 2022; combined heat spike and pesticide esfenvalerate: Delnat et al., 2022). The mechanisms at the origin of *Daphnia* genotype sensitivity to environmental factors have also been suggested to be both genetic and epigenetic, as explored by Asselman et al. (2015), and J. Kim et al. (2023).

b. Epigenetic regulation

In every exposure scheme to MIT, a few DEGs stood out as they simultaneously marked MIT exposure in all lineages (or nearly). 25 out of the 26 of them are lncRNAs, which are indeed emerging as key regulators of multiple biological processes in eukaryotes, and principally in reproduction and developmental processes (Valenzuela-Miranda et al., 2017). In daphnia, some lncRNAs are responsible for dimorphic gene expression (*Dsx1*), and even provoke obligate asexuality of some lines (Kato et al., 2018; Dane et al., 2020). It should be noted that as MIT stress did not provoke a transition to sexual reproduction, any potential link to lncRNAs in this context remains speculative. Because not only they can regulate gene expression through post-transcriptional processing, like micro RNAs, but they can also regulate protein production through chromatin remodelling and transcriptional control (Mercer et al., 2009). Their wide range of action justifies the multiple regulatory biological processes in which they can be involved in addition to the aforementioned reproduction and development, such as metabolism and immune response (Imamura & Akimitsu, 2014; Zhao & Lin, 2015). These findings provide a better understanding of the potential molecular mechanisms underlying the effects of MIT exposure on non-target species, highlighting the need for a more comprehensive approach to

ecotoxicology that incorporates molecular and evolutionary perspectives. For in-depth comprehension, a more precise sampling, at the organ level for instance, might be as useful as it has been in vertebrates to reveal information of environmental disturbance (Koenig et al., 2021; Zafar et al., 2023). Hopefully, the results will someday also enrich the very little number of entries in the lncRNAdb⁵³.

Overall, the work presented in this thesis sets solid quantitative bases for the study of MIT toxicity on *Daphnia pulex*. It adds to the growing body of evidence that not only do contaminants such as MIT induce a physiological stress, which act differently upon organisms of the same or of different species, but they also may have evolutionary impact on populations in the form of selective pressure, that can ripple onto communities, and by extension ecosystem functioning. The simultaneous test of multigenerational and both high and low dose exposures provided interesting insights into acclimation mechanisms. Moreover, the complementarity of phenotypic and transcriptomic scales revealed cryptic transgenerational effects. The mechanisms of toxicity identified via the water flea responses were generalist, as expected considering the properties and mode of action of the molecule.

⁵³ <https://rnacentral.org/expert-database/lncrnadb>

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY MATERIAL – CHAPTER 1

Study clones: populations of origin

Table S1-1. Locations of the *D. pulex* study populations of origin of the study's clonal lineages

Clonal line		Population origin			Isolation date
Name	Code	Type	Location		
Séné 2	SE2	Natural population	Séné	47.616636, -2.713638	10/2019
Séné 5	SE5	Natural population	Séné	47.616636, -2.713638	10/2019
Pearl 7	PE7	Aquaculture tanks	Rennes	48.112800, -1.709124	10/2019
Pearl 16	P16	Aquaculture tanks	Rennes	48.112800, -1.709124	10/2019
Rennes	RE0	Aquaculture tanks	Rennes	48.112800, -1.709124	12/2016
Goven 6	GO6	Natural population	Goven	47.994867, -1.859119	10/2019
Lassalle	LA0	Natural population	Saint-Judoce	48.359723, -1.951767	11/2019
Alsace	AL0	Commercial strain (Aqualiment©)	-	-	bought in 2016

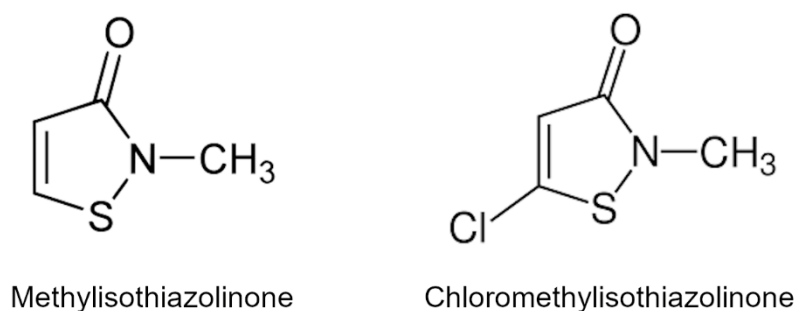


Figure S1-1. Formulas of 2-méthylisothiazol-3(2H)-one (CAS n°2682-20-4) and 5-Chloro-2-methyl-1,2-thiazol-3(2H)-one (CAS n°26172-55-4)

Calibration curves were obtained from standards at the following concentrations: (1) 0.02, 0.04, 0.2, 0.6 and 0.8 mg/L and 0.2 mg/L of the internal standard solution for the mixture of CMIT and MIT and at (2) 0.1, 0.2, 1.5, 5.5 and 6.5 mg/L and 1.5 mg/L of the internal standard solution for the MIT alone. The concentrations reported for the mixture are the cumulated concentrations of both CMIT and MIT (3:1 ratio). Each solution and blanks were injected ten times.

Using quantification transitions, CMIT and MIT areas were normalized by the I.S. area for each injection (calibration and bioassay samples). Calibration curves (Figure 1) were generated with R (version 4.0.0) as linear regression with generalized least squares to correct for heteroscedasticity of residuals.

Table S1-2. Mass transitions and retention times for the target compounds in UPLC-MS/MS. I.S., internal standard.

Compound	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	Collision energy (eV)	Retention time (min)
MIT	116.0170	100.9935	28	0.3 – 0.4
CMIT	149.9790	134.9546	28	1.1 – 1.2
MIT-D3 (I.S.)	119.0360	100.9935	28	0.3 – 0.4

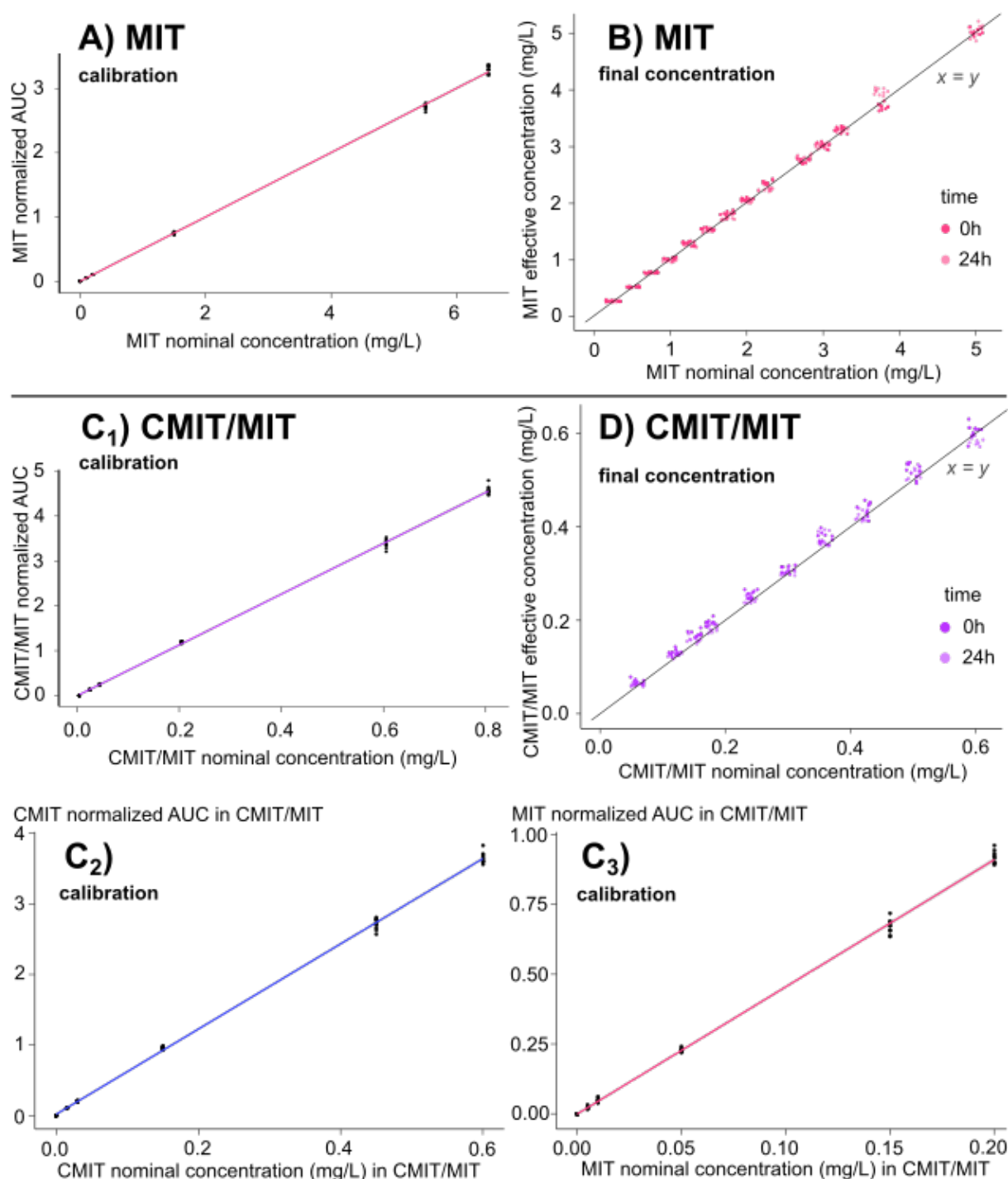


Figure S1-2. Measures of isothiazolinones concentration in water contaminated with MIT (A and B) or CMIT/MIT (C₁₋₂₋₃ and D), with 10 technical replicates. In calibration curves (A and C), the areas under the curve (AUC) are normalized by the internal standard area. For CMIT/MIT samples, calibration curves show CMIT (C₂, adjusted $R^2 = 0.999$, p-value < 0.001) and MIT (C₃, adjusted $R^2 = 0.998$, p-value < 0.001) AUC separately, and the mixture CMIT/MIT (C₁, adjusted $R^2 = 0.999$, p-value < 0.001) as derived from the single molecules' AUC. Based on calibration curves, the bioassay samples' effective concentrations are plotted against nominal concentrations for MIT samples (B) and CMIT/MIT samples (D), showing strong linear relationship in both cases (MIT: $R^2 = 0.998$, p-value < 0.001; CMIT/MIT: $R^2 = 0.995$, p-value < 0.001). Effective concentrations in samples are displayed at 0 and 24h after contamination and are reported in supplemental material (table S3).

Table S1-3. CMIT and MIT concentrations measured with analysis by UPLC-MS/MS (mg/L) and degradation over 24h. CMIT+MIT concentration was computed by adding CMIT and MIT measures in mixture.

	Nominal concentration (mg/L)	Effective median concentration in mg/L (SD)	Effective median concentration in mg/L (SD) at 0h	Effective median concentration in mg/L (SD) at 24h	Percentage decrease in concentration over 24h
CMIT+MIT in mixture	0.06	0.064 (0.005)	0.067 (0.005)	0.062 (0.002)	7.5 %
	0.12	0.129 (0.006)	0.131 (0.006)	0.126 (0.004)	3.8 %
	0.15	0.166 (0.009)	0.170 (0.006)	0.158 (0.006)	7.1 %
	0.18	0.191 (0.009)	0.195 (0.006)	0.182 (0.007)	6.7 %
	0.24	0.248 (0.009)	0.253 (0.008)	0.244 (0.007)	3.6 %
	0.30	0.305 (0.007)	0.311 (0.007)	0.303 (0.007)	2.6 %
	0.36	0.370 (0.012)	0.376 (0.011)	0.366 (0.014)	2.7 %
	0.42	0.431 (0.015)	0.437 (0.016)	0.423 (0.014)	3.2 %
	0.50	0.519 (0.014)	0.52 (0.016)	0.519 (0.012)	0.2 %
	0.60	0.595 (0.016)	0.608 (0.015)	0.585 (0.012)	3.8 %
CMIT in mixture	0.045	0.047 (0.004)	0.049 (0.005)	0.045 (0.002)	8.2 %
	0.090	0.093 (0.004)	0.094 (0.005)	0.091 (0.004)	3.2 %
	0.112	0.118 (0.006)	0.122 (0.005)	0.114 (0.005)	6.6 %
	0.135	0.137 (0.007)	0.139 (0.005)	0.130 (0.005)	6.5 %
	0.180	0.177 (0.007)	0.180 (0.007)	0.175 (0.006)	2.8 %
	0.225	0.219 (0.007)	0.222 (0.006)	0.217 (0.007)	2.3 %
	0.270	0.265 (0.007)	0.269 (0.01)	0.261 (0.012)	3.0 %
	0.315	0.305 (0.012)	0.308 (0.012)	0.302 (0.012)	1.9 %
	0.375	0.370 (0.011)	0.369 (0.012)	0.371 (0.009)	-0.5 %
	0.450	0.424 (0.013)	0.430 (0.013)	0.416 (0.009)	3.3 %
MIT in mixture	0.015	0.019 (0.001)	0.019 (0.001)	0.018 (0.001)	5.3 %
	0.030	0.037 (0.002)	0.039 (0.002)	0.036 (0.001)	7.7 %
	0.038	0.048 (0.003)	0.051 (0.002)	0.047 (0.002)	7.8 %
	0.045	0.055 (0.003)	0.058 (0.002)	0.054 (0.003)	6.9 %
	0.060	0.075 (0.003)	0.076 (0.002)	0.072 (0.002)	5.3 %
	0.075	0.092 (0.002)	0.092 (0.002)	0.090 (0.002)	2.2 %
	0.090	0.112 (0.003)	0.112 (0.003)	0.112 (0.003)	0.0 %
	0.105	0.129 (0.004)	0.132 (0.004)	0.127 (0.002)	3.8 %
	0.125	0.155 (0.004)	0.156 (0.004)	0.154 (0.004)	1.3 %
	0.150	0.179 (0.006)	0.181 (0.006)	0.176 (0.004)	2.8 %
MIT	0.25	0.258 (0.110)	0.260 (0.011)	0.255 (0.011)	1.9 %
	0.5	0.507 (0.009)	0.505 (0.009)	0.511 (0.007)	-1.2 %
	0.75	0.764 (0.100)	0.767 (0.011)	0.759 (0.009)	1.0 %
	1.0	0.993 (0.027)	1.012 (0.012)	0.969 (0.015)	4.2 %
	1.25	1.255 (0.034)	1.291 (0.018)	1.233 (0.012)	4.5 %
	1.5	1.520 (0.026)	1.531 (0.016)	1.495 (0.025)	2.4 %
	1.75	1.762 (0.055)	1.801 (0.026)	1.707 (0.021)	5.2 %
	2.0	2.038 (0.026)	2.054 (0.022)	2.030 (0.024)	1.2 %
	2.25	2.305 (0.069)	2.341 (0.030)	2.226 (0.031)	4.9 %
	2.75	2.759 (0.048)	2.715 (0.034)	2.780 (0.044)	-2.4 %
	3.0	3.016 (0.041)	2.996 (0.048)	3.021 (0.033)	-0.8 %
	3.25	3.283 (0.040)	3.296 (0.048)	3.278 (0.033)	0.5 %
	3.75	3.827 (0.146)	3.685 (0.050)	3.955 (0.051)	-7.3 %
	5.0	4.996 (0.085)	4.996 (0.049)	4.996 (0.113)	0 %

Survival data

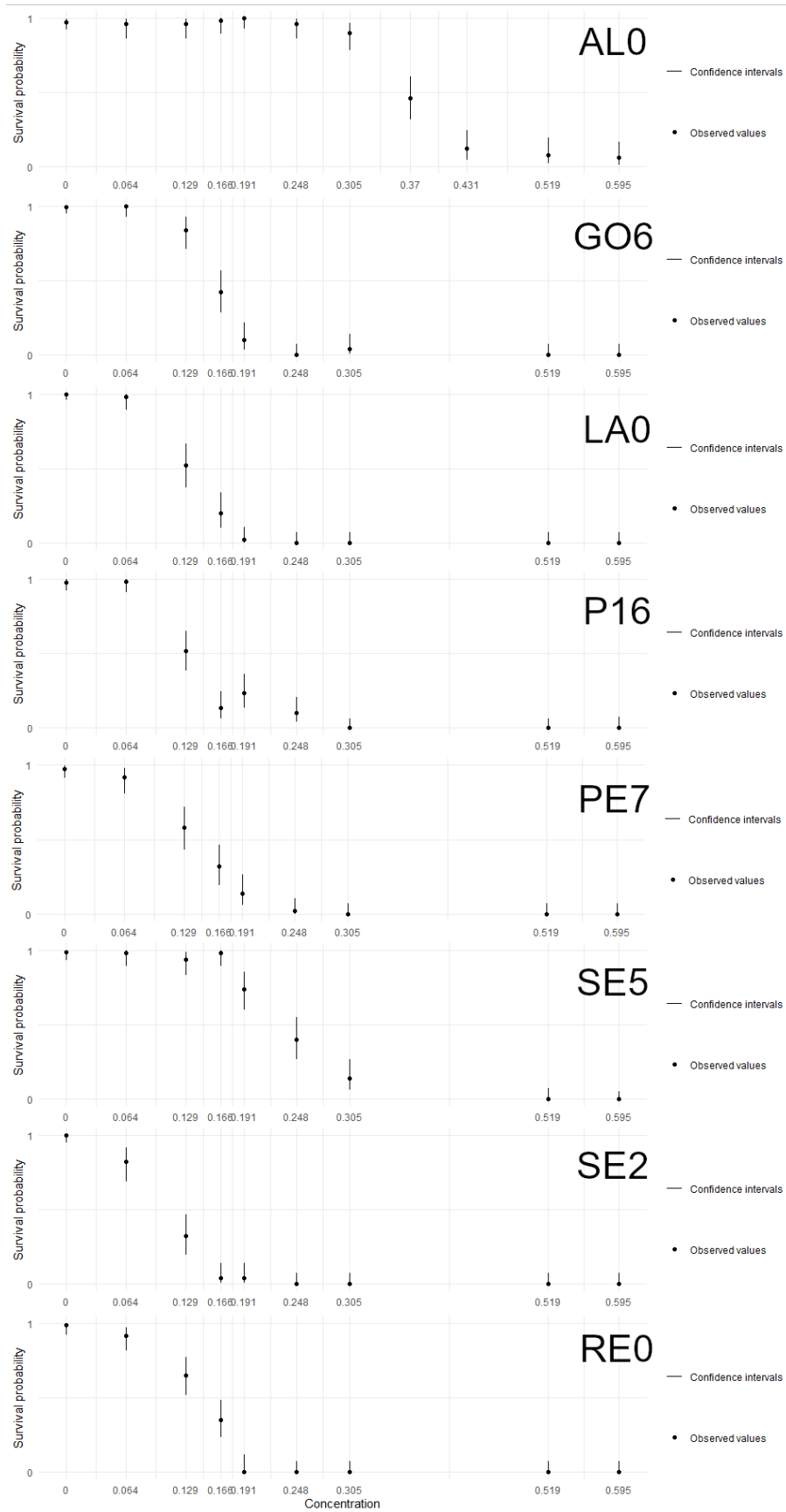


Figure S1-3. Survival rate of each clonal line for the CMIT/MIT assay at 48h as a function of concentration, with binomial confidence intervals around the data (n=50).

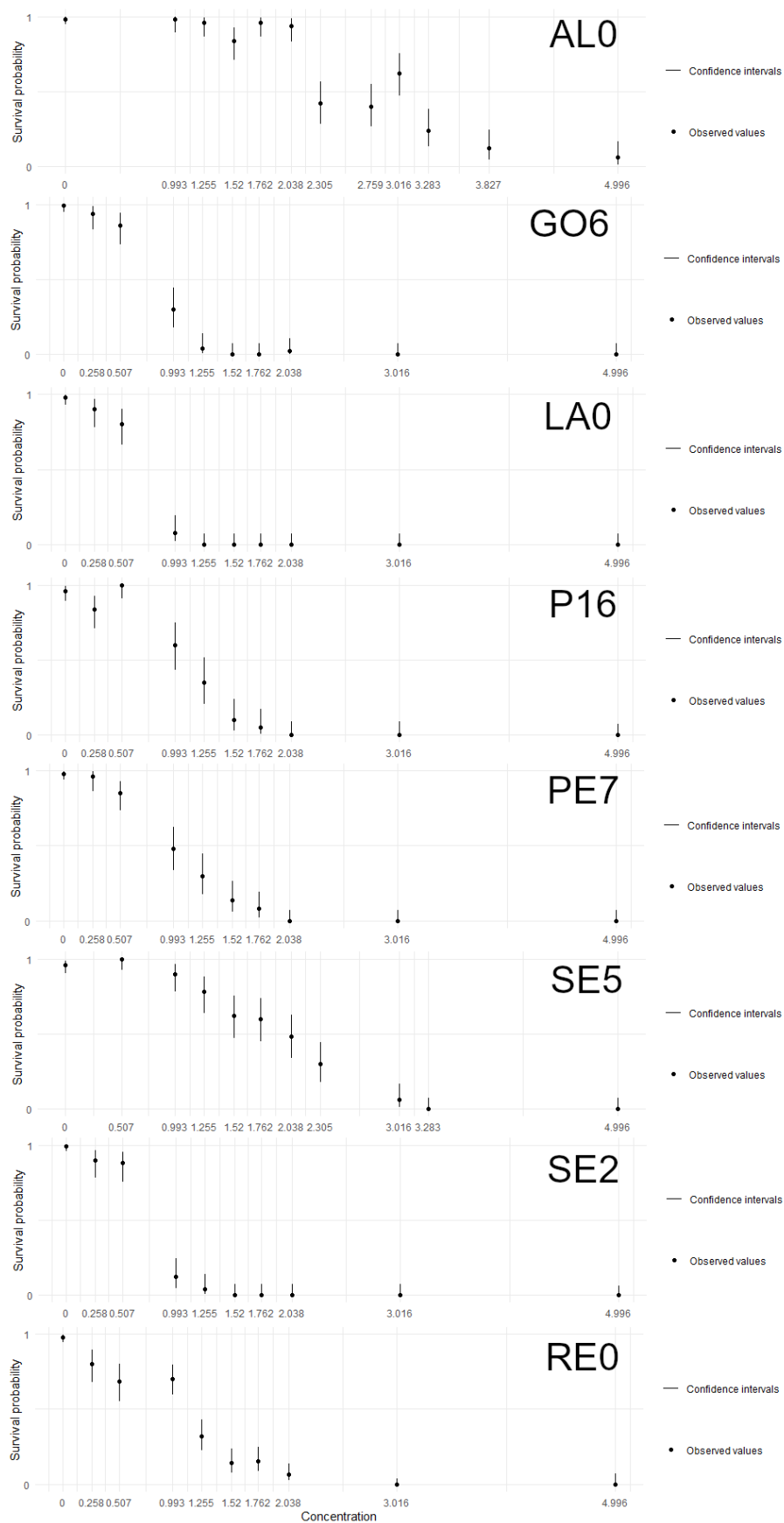


Figure S1-4. Survival rate of each clonal line for the MIT assay at 48h as a function of concentration, with binomial confidence intervals around the data (n=50).

Modeling

Table S1-4. GUTS models parameters estimates for the 8 clonal lineages, median and 95% credible interval. With general GUTS parameters: h_b as the background mortality, and k_D as the dominant rate constant, that is to the slowest compensating process dominating the overall dynamics of toxicity. Parameters specific of IT models are related to the individual threshold concentration which is described as a log-logistic probability distribution with m_w the median and β the shape of the distribution

Clone	CMIT + MIT				MIT			
	k_D	h_b (e-02)	m_w	β	k_D	h_b (e-02)	m_w	β
Alsace	1.25 [1.01-1.51]	1.09 [0.49-2.06]	0.34 [0.31-0.37]	6.62 [5.52-7.85]	0.98 [0.72-1.25]	0.81 [0.23-1.92]	2.44 [2.10-2.71]	3.32 [2.77-3.92]
Goven6	0.93 [0.68-1.18]	0.37 [0.05-1.29]	0.13 [0.11-0.15]	5.82 [4.85-6.95]	1.53 [1.12-2.02]	1.22 [0.28-2.97]	0.74 [0.63-0.86]	5.38 [4.24-7.07]
Lassalle	1.31 [0.98-1.67]	0.25 [0.02-1.31]	0.12 [0.10-0.13]	5.17 [4.18-6.36]	0.75 [0.46-1.10]	2.64 [1.00-5.03]	0.52 [0.37-0.66]	5.62 [4.39-7.13]
Rennes	1.82 [1.41-2.28]	1.32 [0.19-3.74]	0.14 [0.12-0.15]	4.89 [3.82-6.33]	0.94 [0.60-1.29]	2.52 [0.81-5.24]	0.81 [0.58-1.02]	2.58 [2.03-3.47]
Sene2	1.23 [0.87-1.63]	0.35 [0.02-2.17]	0.09 [0.07-0.10]	4.22 [3.46-5.19]	0.97 [0.65-1.36]	1.70 [0.67-3.36]	0.63 [0.49-0.76]	6.66 [5.32-8.46]
Sene5	1.69 [1.35-2.07]	0.74 [0.15-2.04]	0.23 [0.21-0.25]	5.71 [4.69-6.92]	1.51 [1.22-1.82]	1.62 [0.60-3.38]	1.75 [1.60-1.89]	4.66 [3.92-5.54]
Pearl16	1.28 [0.97-1.62]	0.76 [0.17-2.21]	0.12 [0.10-0.13]	3.64 [3.05-4.31]	0.66 [0.39-0.94]	3.35 [1.72-5.71]	0.79 [0.54-0.98]	4.86 [3.90-6.00]
Pearl7	1.46 [1.08-1.85]	1.31 [0.28-3.46]	0.13 [0.11-0.14]	4.27 [3.43-5.36]	0.62 [0.36-0.91]	1.15 [0.35-2.64]	0.66 [0.44-0.84]	3.59 [3.01-4.31]

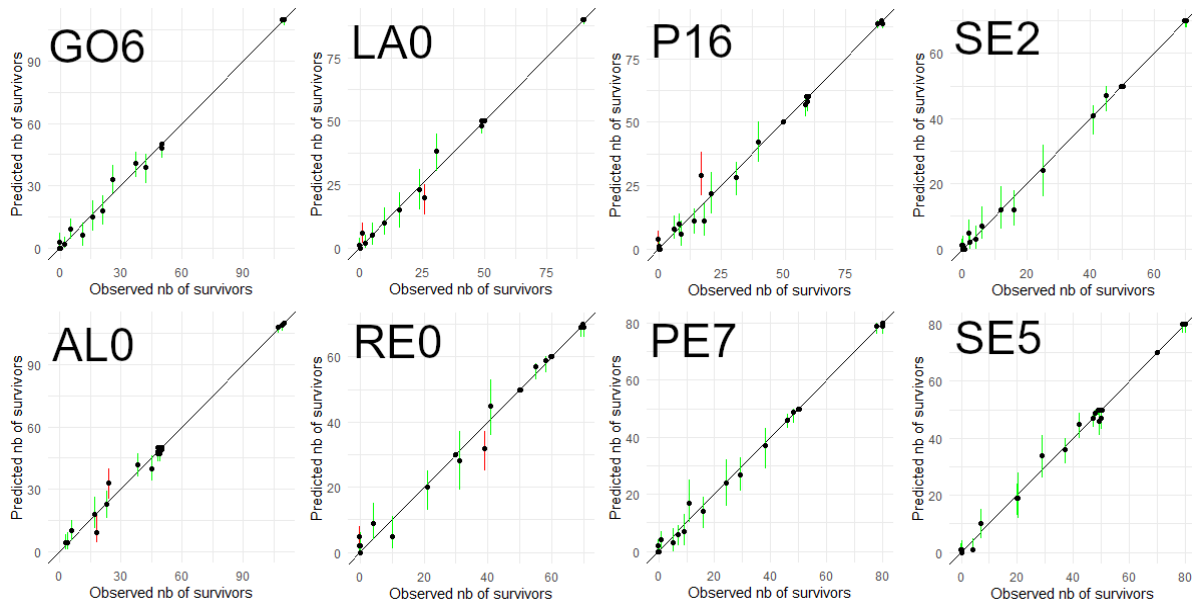


Figure S1-5. Posterior predictive checks of GUTS-RED-IT models for the eight clonal lineages for CMIT/MIT assay. The dots represent the median values of the predicted number of survivors and segments are the 95% credible intervals, and the x-axis correspond to the observed values. The segments are green when the observed value falls in the 95% credible interval and red otherwise.

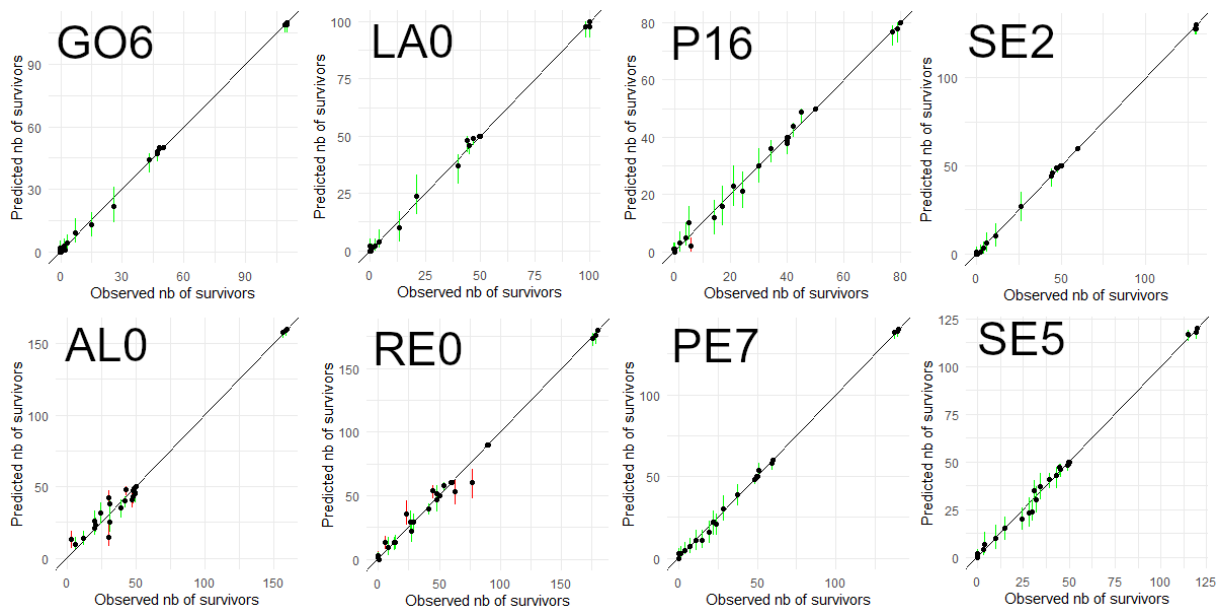


Figure S1-6. Posterior predictive checks of GUTS-RED-IT models for the eight clonal lineages for MIT assay. The dots represent the median values of the predicted number of survivors and segments are the 95% credible intervals, and the x-axis correspond to the observed values. The segments are green when the observed value falls in the 95% credible interval and red otherwise.

Time-target model

Source: morse modelling vignette (<https://CRAN.R-project.org/package=morse>)

The number N_i of surviving organisms at time t follows a binomial distribution:

$$N_i B(n_i^{init}, f(c_i))$$

With n_i^{init} as the initial number of organisms and c the contaminant level, and f the three parameters log-logistic function:

$$f(c) = \frac{d}{1 + \left(\frac{c}{e}\right)^b}$$

Where b relates to the effect intensity of the contaminant and so to the steepness of the slope in the dose-response curve, d is the survival rate in absence of contaminant, and e is the LC_{50} .

Table S1-5. Log-logistic models parameters estimates at 48h for the 8 clonal lineages, median and 95% credible interval. The parameters describe as follow: b relates to the effect intensity of the contaminant and so to the steepness of the slope in the dose-response curve, d is the survival rate in absence of contaminant, and e is the LC_{50} .

Clone	CMIT + MIT			MIT		
	b	d	e	b	d	e
Alsace	8.69 [6.92-10.83]	0.97 [0.95-0.99]	0.37 [0.36-0.39]	4.71 [3.87-5.74]	0.98 [0.95-0.99]	2.73 [2.59-2.88]
Goven6	8.16 [6.23-10.54]	0.99 [0.97-1.00]	0.157 [0.148-0.164]	5.48 [4.10-8.61]	0.97 [0.93-0.99]	0.79 [0.70-0.92]
Lassalle	7.09 [5.34-9.34]	-	0.13 [0.12-0.14]	6.84 [5.02-16.54]	0.95 [0.90-0.98]	0.66 [0.59-0.86]
Rennes	8.11 [5.72-10.98]	0.95 [0.90-0.98]	0.146 [0.137-0.154]	4.33 [2.79-5.81]	0.90 [0.86-0.95]	1.14 [0.96-1.24]
Sene2	4.45 [3.54-5.51]	-	0.10 [0.09-0.11]	6.63 [4.85-10.93]	0.96 [0.93-0.99]	0.74 [0.65-0.86]
Sene5	7.36 [5.71-9.51]	0.98 [0.95-0.99]	0.24 [0.22-0.25]	4.81 [3.91-6.01]	0.96 [0.92-0.98]	1.86 [1.74-1.98]
Pearl16	4.68 [3.72-5.87]	0.98 [0.94-0.99]	0.13 [0.12-0.14]	6.76 [5.01-8.99]	0.93 [0.88-0.96]	1.12 [1.03-1.20]
Pearl7	5.86 [4.22-7.93]	0.95 [0.91-0.99]	0.14 [0.13-0.15]	4.01 [3.12-5.38]	0.97 [0.93-0.99]	0.96 [0.85-1.07]

Table S1-6. Fixed-effects coefficients and variance estimates of random-effects for survival modelled as a function of line-specific response to dosage.

CMIT/MIT				
Model Coeff.	Est.	PDI_{0.025}	PDI_{0.975}	pMCMC
β_0 ALS	6.33	5.58	7.17	0.001
β_0 GOV	8.37	6.60	10.11	0.001
β_0 LAS	8.60	6.74	10.53	0.001
β_0 PEA	4.92	2.91	6.98	0.032
β_0 REN	5.82	3.91	7.73	0.386
β_0 SEN2	5.93	3.99	7.88	0.522
β_0 SEN5	6.83	5.03	8.99	0.426
β_0 U3E7	6.62	4.60	8.63	0.674
β_1 ALS	-16.35	-19.35	-13.90	0.001
β_1 GOV	-55.73	-62.00	-49.75	0.001
β_1 LAS	-70.78	-78.60	-62.43	0.001
β_1 PEA	-37.72	-45.93	-30.14	0.001
β_1 REN	-46.30	-53.42	-40.15	0.001
β_1 SEN2	-58.99	-65.60	-52.79	0.001
β_1 SEN5	-29.70	-36.95	-22.72	0.001
β_1 U3E7	-53.48	-60.35	-46.78	0.001
Var. Comp.	Var	Var_{0.025}	Var_{0.975}	
Var{ β_0 Rep Line}	1.7370	1.3870	2.1330	
Var{residual}	0.0592	0.0003	0.2070	
MIT				
Model Coeff.	Est.	PDI_{0.025}	PDI_{0.975}	pMCMC
β_0 ALS	5.41	4.33	6.34	0.001
β_0 GOV	5.07	2.56	7.39	0.612
β_0 LAS	4.13	1.92	6.26	0.046
β_0 PEA	4.41	1.91	6.58	0.122
β_0 REN	4.02	1.78	6.03	0.016
β_0 SEN2	4.20	1.62	6.63	0.150
β_0 SEN5	5.05	2.77	7.19	0.560
β_0 U3E7	4.72	2.48	7.13	0.356
β_1 ALS	-1.90	-2.33	-1.55	0.001
β_1 GOV	-6.51	-8.22	-5.44	0.001
β_1 LAS	-6.50	-8.35	-5.41	0.001
β_1 PEA	-4.58	-5.77	-3.53	0.001
β_1 REN	-3.95	-4.83	-3.09	0.001
β_1 SEN2	-5.88	-8.05	-4.28	0.001
β_1 SEN5	-2.86	-3.86	-2.00	0.004
β_1 U3E7	-4.73	-6.19	-3.80	0.001
Var. Comp.	Var	Var_{0.025}	Var_{0.975}	
Var{ β_0 Rep Line}	2.0370	1.4790	2.7080	
Var{residual}	0.0509	0.0013	0.3011	

A. METHODS

Parental generation survival

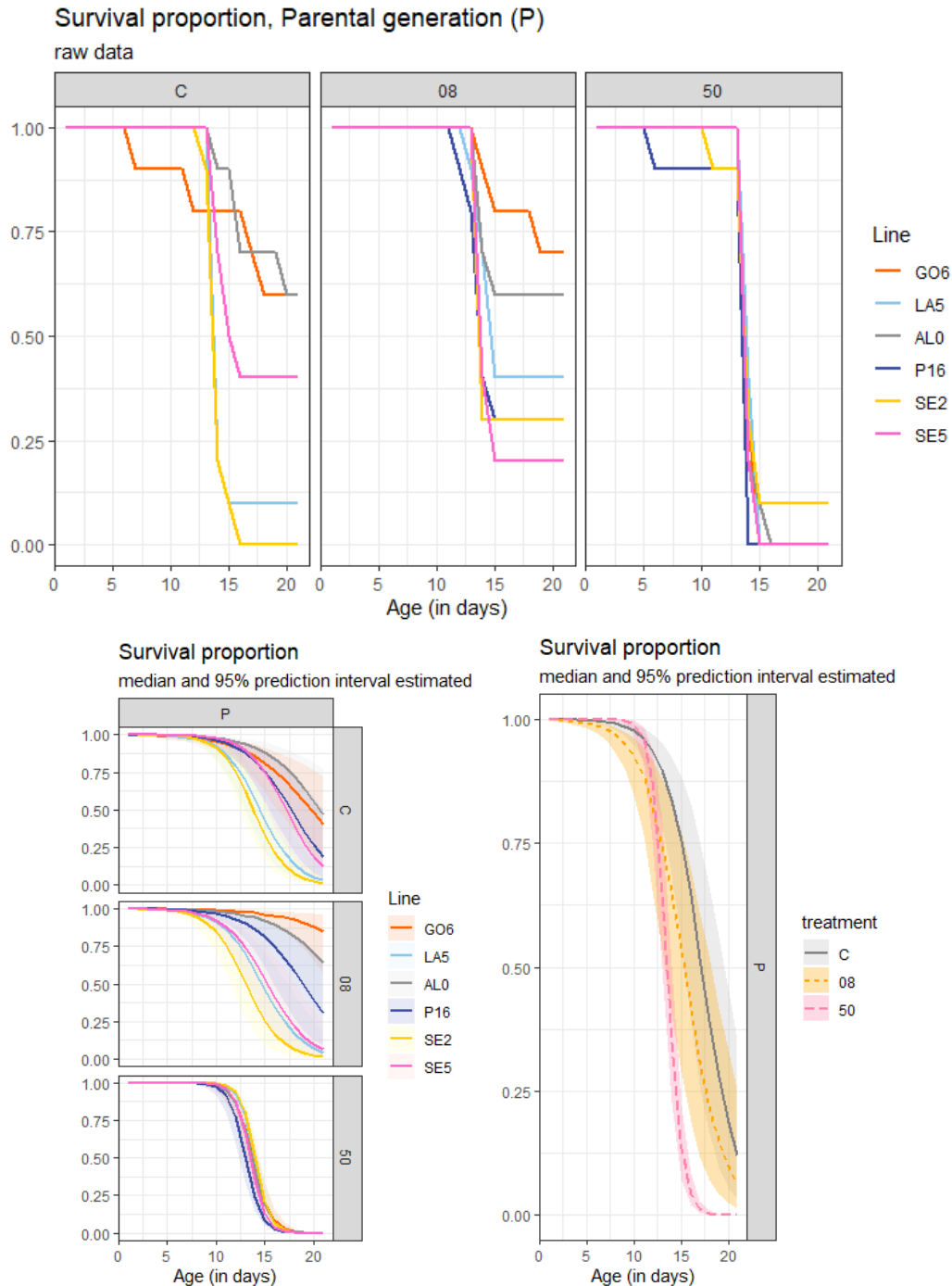


Figure S2-1. Survival data for the P generation, raw (top panel) or estimated from GLMM (bottom panels). The high mortality in controls (all clones) revealed poor conditions, which were therefore adapted in following generations.

Water quality monitoring

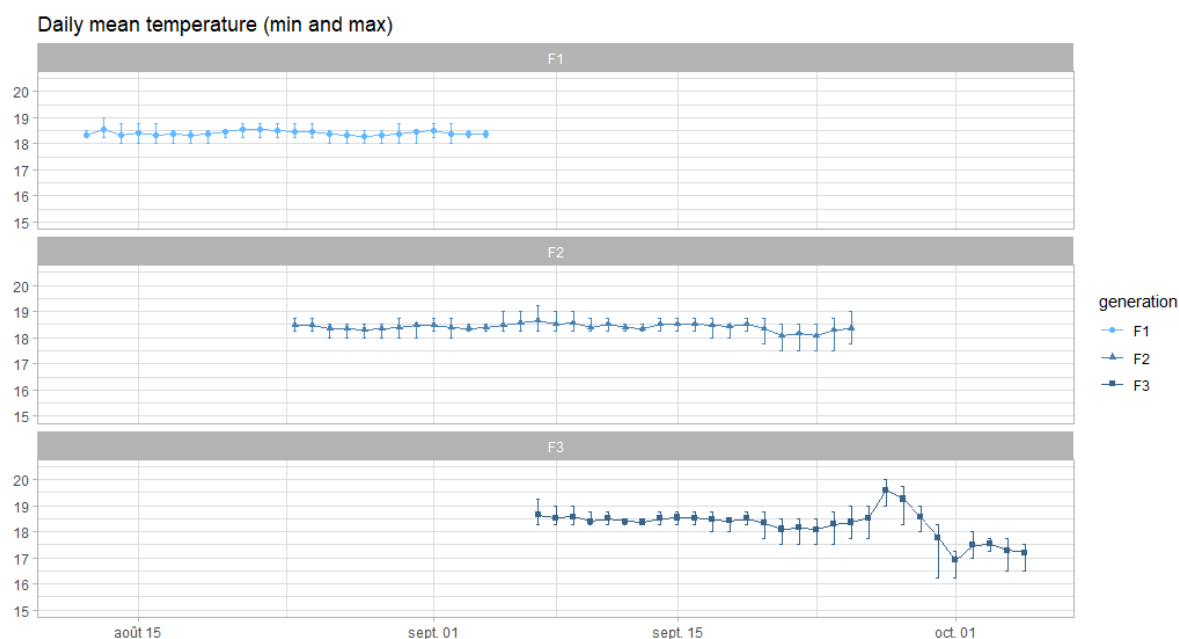


Figure S2-2. Daily water temperature (°C) recorded during the three generations of daphnia used in the lifetable experiment. Dots (circles, triangles, and squares) indicate mean-values, and error bars mark the min and max temperature recorded. Temperature was quite stable in F1, F2 and during the 20 first days of F3 survey (mostly < 1°C). By contrast, from the date of September 27th, thermal variation intensified, which affected some F3 conditions (see methods 2.4.1).

Table S2-1. Weekly water quality control including pH, dissolved oxygen (in mg and in percentage), conductivity and temperature.

day	pH	O2_mgL	O2_%	Conductivity (μS/cm)	Temperature (°C)
28/07/2021	8.369	9.30	100.1	523	18.6
04/08/2021	8.079	9.13	98.3	527	18.7
11/08/2021	8.578	9.93	107.3	526	18.8
18/08/2021	8.262	9.01	96.5	505	18.8
25/08/2021	8.177	9.19	98.3	482	18.7
01/09/2021	8.245	9.37	99.5	532	18.6
08/09/2021	8.307	9.17	99.4	540	18.8
15/09/2021	8.195	9.38	101.2	531	18.7
23/09/2021	8.209	9.48	100.6	497	18.5
29/09/2021	8.537	9.56	102.3	495	18.8

Daphnia body size



Figure S2-3. *Daphnia* body size was measured at age 1 (left) and 21 days (right); their length was measured from the centre of the eye to the base of the spine (distance between purple arrows). For each individual photographed, the measure of distance with imageJ was repeated 4 times and averaged to get a final value of length.

MIT effective concentration

Effective concentrations were measured over 7 days to test for significant degradation, with UPLC and 2 different mass spectrometers (either QToF or TQD). We separated the experiments between 2 datasets: ‘MG50’ (10 - 90 µg/L) and ‘MG08’ (0.1-1.6 µg/L).

Table S2-2. Mass transitions and retention times for the target compounds in UPLC-MS/MS. I.S., internal standard.

Compound	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)	Retention time (min)
MIT	116.0170	100.9935	28	0.3 – 0.4
MIT-D3 (I.S.)	119.0360	100.9935	28	0.3 – 0.4

1. Internal standard (d3-MIT) signal (stability desired)

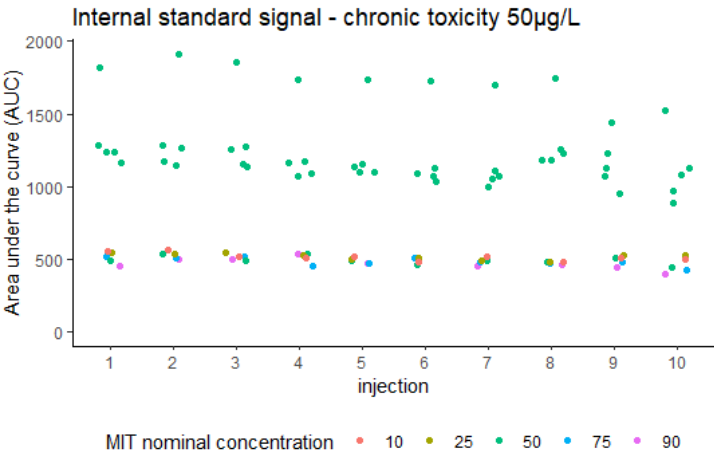


Figure S2-4. Internal standard (d3-MIT) signal in MG50 dataset samples, TQD MS. All AUC from each MIT nominal concentration in the 10 injections (replicates) are similar, allowing the use of the full dataset for calibration and quantification of MIT.

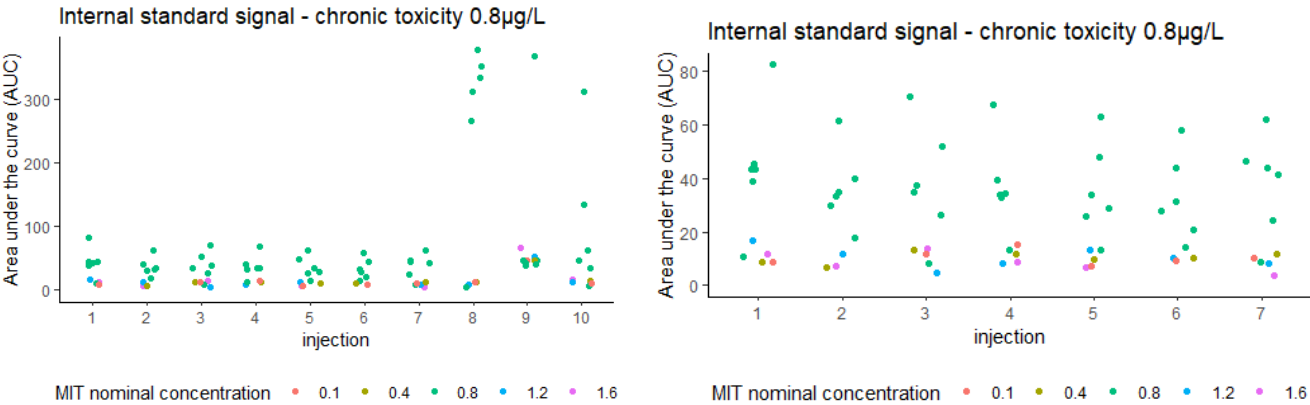


Figure S2-5. Internal standard (d3-MIT) signal in MG08 dataset samples, TQD MS. Given the high variation in injections 8 to 10 as compared to other replicates (left), these were dismissed from further analyses. These variations may be due to a technical issue (low solvent level) more visible at low concentrations than at higher concentrations since replicates 8 to 10 for MG50 samples do not show such variation from the other injections (Figure S2-4). The panel on the right show the data with the first 7 injections only which were those used for calibration and quantification of MIT.

2. Calibration curves

a. MG50 samples

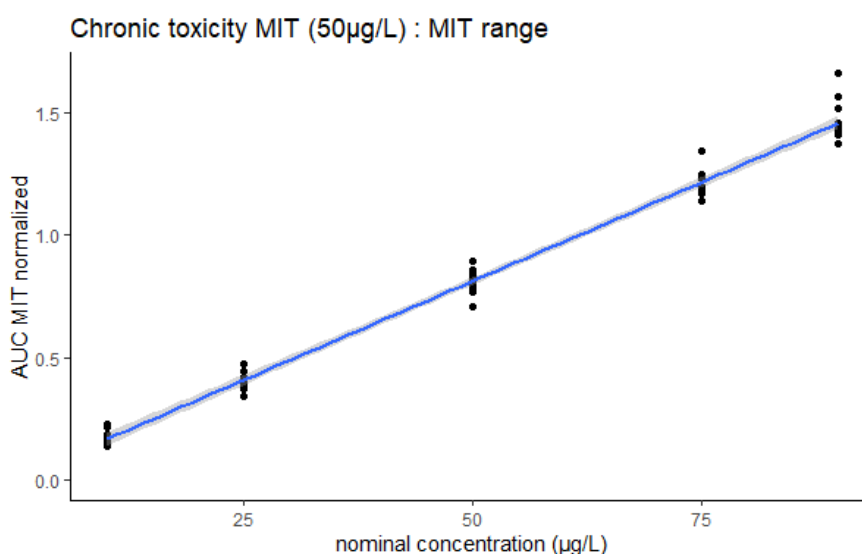


Figure S2-6a. Calibration curve for MG50 samples (adjusted $R^2=0.996$, p-value <0.001). MIT area under the curves (AUC) are normalized by the internal standard's AUC. 10 injections per sample, TQD MS.

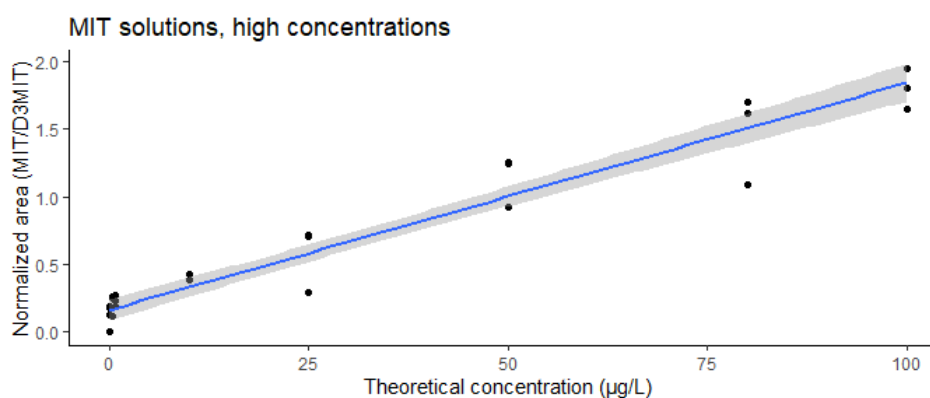


Figure S2-6b. Calibration curve for MG50 samples with QToF MS (adjusted $R^2=0.935$, p-value <0.001). MIT area under the curves (AUC) are normalized by the internal standard's AUC. 3 injections per sample.

a. MG08 samples

The sensitivity of the QToF MS did not allow for quantification of samples with nominal concentrations of 1ppb (1 µg/L) and below. Figure S2-6d show the calibration curve for the MG08 samples and how the method was inappropriate for such low concentrations.

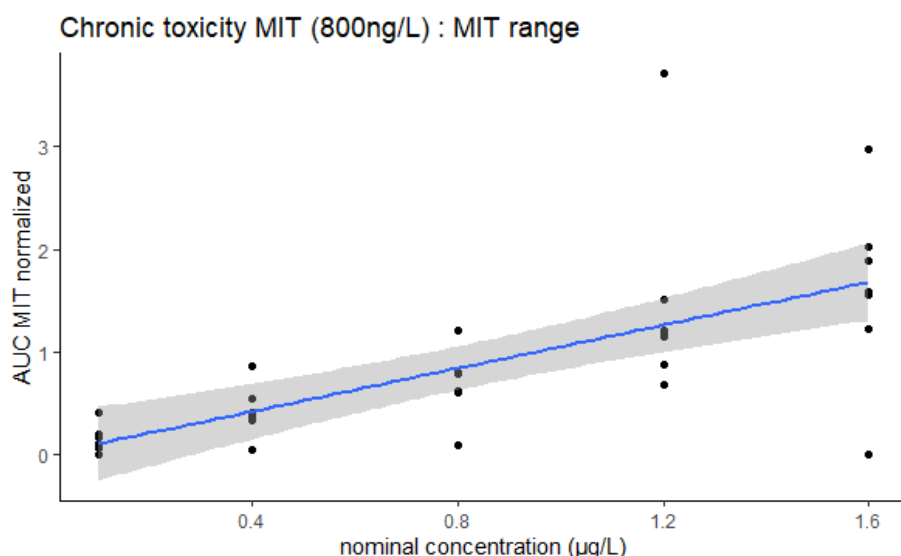


Figure S2-6c. Calibration curve for MG08 samples (adjusted $R^2=0.738$, $p\text{-value}<0.001$). MIT area under the curves (AUC) are normalized by the internal standard's AUC. 7 injections per sample, TQD MS. The quality is less than ideal, because of the very low concentrations tested.

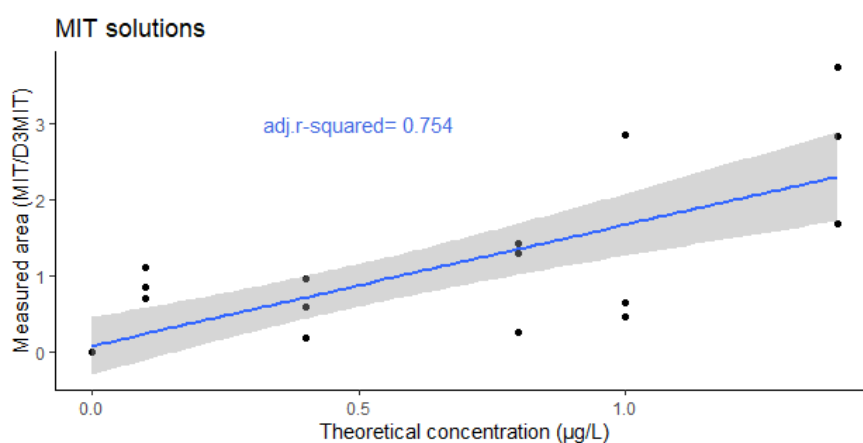


Figure S2-6d. Calibration curve for MG08 samples with QToF MS (adjusted $R^2=0.754$, $p\text{-value}<0.001$). MIT area under the curves (AUC) are normalized by the internal standard's AUC. 3 injections per sample. This dataset was not used in further analyses.

3. Predicted MIT concentrations

Effective concentrations seems lower than expected, with initial mean MIT concentration for MG50 samples of 24 (± 2) µg/L and of 0.28 (± 0.06) µg/L for MG08 samples, and in both datasets it decreased to half these values. Effective concentration results are reported in table S2-3X and S2-4. As expected, for MG50 samples, TQD gave more precise results than QToF (figure S2-7) which is why we relied on

TQD MS values for effective concentration. The increase in concentration at day 3 (around +35%) however was not explained, and yet occurred in both MG50 and MG08 samples (even with the lower precision on MG08 samples' concentration).

a. MG50 samples

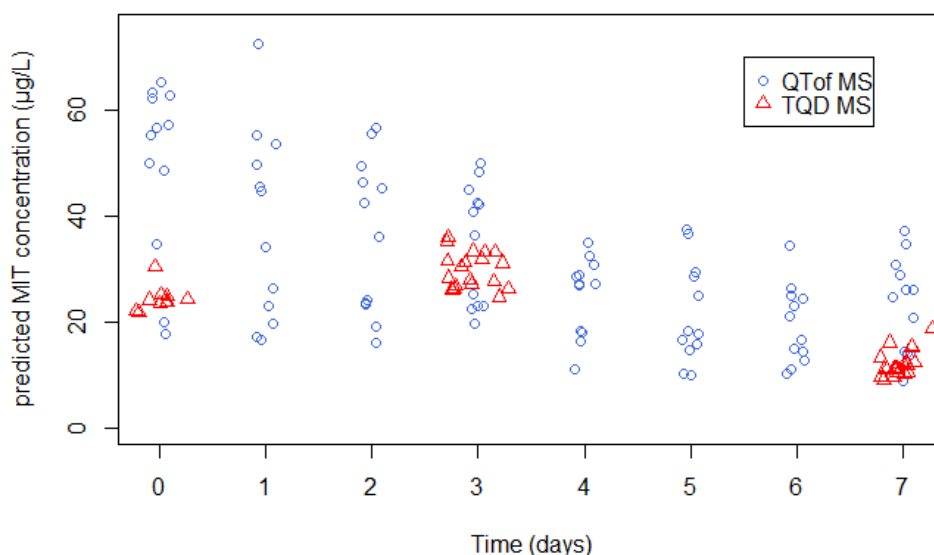


Figure S2-7. MIT concentrations predicted over 7 days with QToF or TQD mass spectrometers for MG50 samples. For the QToF each point represent 1 injection from 4, from 1 of the 3 tested beakers (12 replicates), and for the TQD each point represent 1 injection from 10, of 1 of the 2 jar type tested (20 replicates). TQD MS results were much more precise than those from QToF MS.

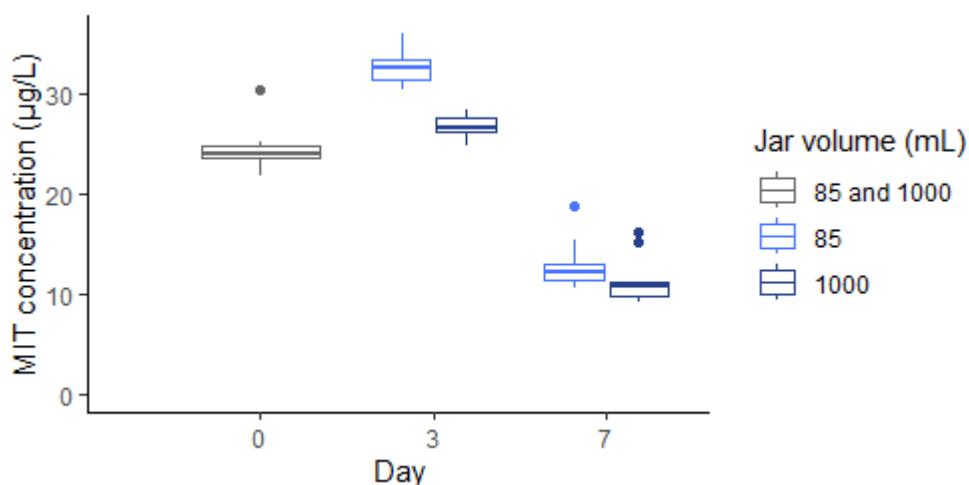


Figure S2-8. MIT concentrations predicted using TDQ-MS over 7 days following initial nominal concentration of 50 µg/L applied to either 1L jars (about 25 daphnia) or 100mL beakers (one single daphnia). 3 samples per type of vessel were mixed into 1 test sample (equal volumes), and 10 injections per tested sample. Within jar types, mean concentrations are statistically different (Kruskal-Wallis $H=40.6$, $p\text{-value}<0.001$ for all vessels; $H=25.6$, $p\text{-value}<0.001$ for 85mL, and $H=23.1$, $p\text{-value}<0.001$ for 1L). On the other hand, differences between jar types were not statistically significant ($H=3.38$, $p=0.184$).

Table S2-3. MIT effective concentrations ($\mu\text{g/L}$) over 7 days, starting with a nominal concentration of $50\mu\text{g/L}$, measured with either QTof (12 replicates) or TQD (20 replicates) MS.

day	QTof MS			TQD MS			
	mean	sd	Ratio to max mean	Mean 85mL	sd 85mL	Mean 1L	sd 1L
0	49	14	1	24	2	24	2
1	38	18	0.77	NA	NA	NA	NA
2	37	11	0.74	NA	NA	NA	NA
3	35	2	0.70	33	2	27	1
4	25	6	0.51	NA	NA	NA	NA
5	22	3	0.44	NA	NA	NA	NA
6	20	1	0.40	NA	NA	NA	NA
7	23	5	0.47	13	2	11	2

b. MG08 samples

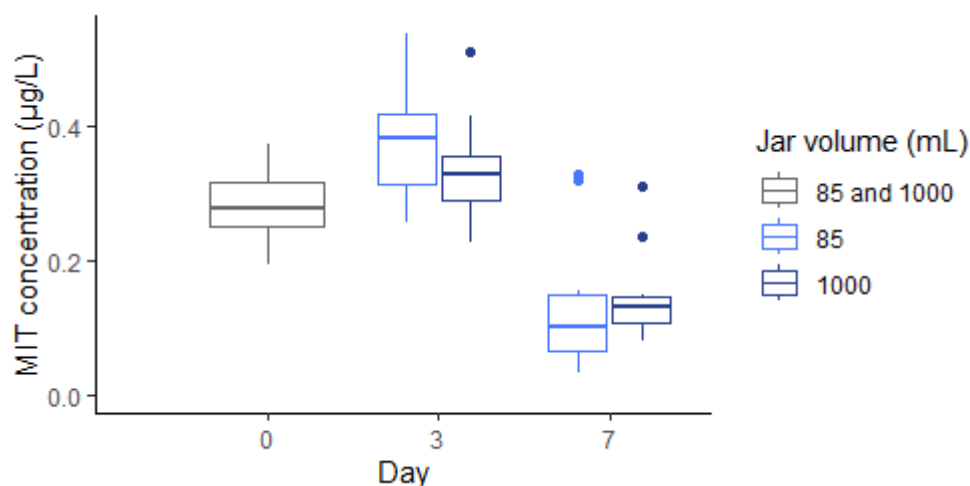


Figure S2-9. MIT concentrations predicted using TDQ-MS over 7 days following initial nominal concentration of $0.8\mu\text{g/L}$ applied to either 1L jars (about 25 daphnia) or 100mL beakers (one single daphnia). 3 vessels per jar type were sampled and mixed into 1 test sample (with equal volumes of each), and 10 injections per tested sample. Within jar types, mean concentrations are statistically different (Kruskal-Wallis $H=15.9$, $p\text{-value}<0.001$ for 85mL, and $H=15.1$, $p\text{-value}<0.001$ for 1L). On the other hand, differences between jar types were not statistically different ($H=0.549$, $p=0.76$).

Table S2-4. MIT effective concentrations over 7 days, starting at a nominal $0.8\mu\text{g/L}$, measured with TQD (20 replicates) mass spectrometer, values in $\mu\text{g/L}$.

day	Mean 85mL	sd 85mL	Mean 1L	sd 1L
0	0.28	0.06	0.28	0.06
3	0.38	0.09	0.34	0.08
7	0.14	0.10	0.15	0.07

B. RESULTS

a. Effects on individual fitness

Empirical QQ plots – individual fitness in F3

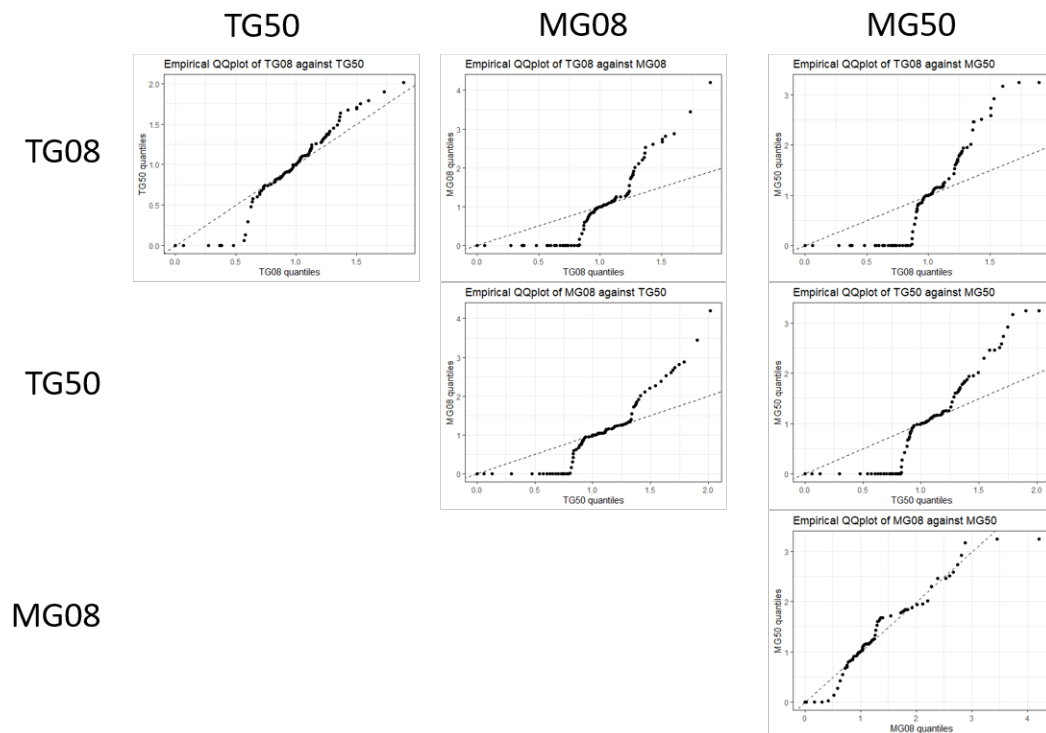


Figure S2-10. Empirical quantile-quantile (QQ) plots of individual fitness values (all dataset) for the four treatment groups (controls excluded, TG: transgenerational; MG: multigenerational; 08: maximal dose 0.8 $\mu\text{g/L}$; 50: maximal dose 50 $\mu\text{g/L}$).

Empirical QQ plots – individual fitness in F3

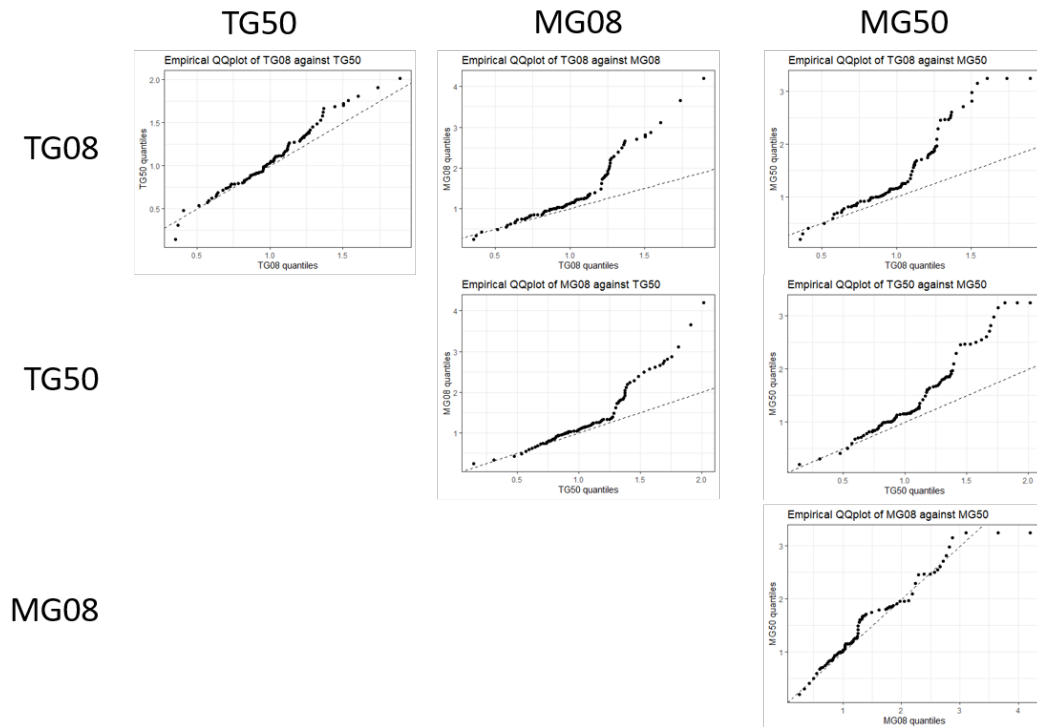


Figure S2-11. Empirical quantile-quantile (QQ) plots of individual fitness values (breeding females) for the four treatment groups (controls excluded, TG: transgenerational; MG: multigenerational; 08: maximal dose 0.8 $\mu\text{g/L}$; 50: maximal dose 50 $\mu\text{g/L}$).

b. Effects on survival

Table S2-5. Comparison of survival-over-time models. Fixed effect structures are constant between models, and include age, treatment, and generation, and the interactions of each other's. Models exhibiting the lowest values of the AIC (Akaike Information Criterion) are determined to represent a parsimony model, explaining the greatest amount of variance relative to model complexity. Treat = treatment, Gen = generation.

Model's random effects structure formulation	Log-Likelihood	df	AIC
β_1 {Age X Line}	-5596	31	11254
β_1 {Treat X Line}	-5402	45	10895
β_1 {Gen X Line}	-5413	36	10899
β_1 {(Age + Treat) X Line}	-5381	51	10864
β_1 {(Age + Gen) X Line}	-5411	40	10902
β_1 {(Treat + Gen) X Line}	-5199	58	10514
β_1 {(Age + Treat + Gen) X Line}	-5180	66	10493

Table S2-6. Fixed effects coefficients and variance estimates of random effects for parsimony model of median age-survival curves.

Model coefficient	Estimates	Standard error
β_0	5.3913	0.4766
β_1 Age	-0.1754	0.0307
β_1 Treatment MG08	-2.4739	0.5598
β_1 Treatment MG50	0.8441	1.1583
β_1 Treatment TG08	-0.5937	0.7531
β_1 Treatment TG50	-2.9289	0.5721
β_1 Generation F2	-1.7000	0.6441
β_1 Generation F3	-1.8031	0.6635
β_1 Age X Treat. MG08	-0.1731	0.0332
β_1 Age X Treat. MG50	0.0420	0.0387
β_1 Age X Treat. TG08	-0.0059	0.0346
β_1 Age X Treat. TG50	-0.1183	0.0320
β_1 Age X Gen. F2	0.0665	0.0318
β_1 Age X Gen. F3	0.0408	0.0315
β_1 Treat. MG08 X Gen. F2	1.1205	0.5645
β_1 Treat. MG50 X Gen. F2	2.1309	0.8057
β_1 Treat. TG08 X Gen. F2	1.7429	0.6929
β_1 Treat. TG50 X Gen. F2	2.8444	0.5819
β_1 Treat. MG08 X Gen. F3	2.5290	0.5830
β_1 Treat. MG50 X Gen. F3	1.8510	0.8044
β_1 Treat. TG08 X Gen. F3	4.2908	0.9455
β_1 Treat. TG50 X Gen. F3	4.9367	0.7005
β_1 Age X Treat. MG08 X Gen. F2	0.0385	0.0392
β_1 Age X Treat. MG50 X Gen. F2	-0.0476	0.0506
β_1 Age X Treat. TG08 X Gen. F2	-0.0090	0.0435
β_1 Age X Treat. TG50 X Gen. F2	0.1187	0.0389
β_1 Age X Treat. MG08 X Gen. F3	0.1553	0.0396
β_1 Age X Treat. MG50 X Gen. F3	-0.0454	0.0500
β_1 Age X Treat. TG08 X Gen. F3	-0.0815	0.0563
β_1 Age X Treat. TG50 X Gen. F3	0.1152	0.0455
Variance components	Variance	Standard Deviation
Var{ β_1 Line Age}	0.0011	0.0328
Var{ β_1 Line Treatment MG08}	0.1159	0.3404
Var{ β_1 Line Treatment MG50}	0.4780	0.6914
Var{ β_1 Line Treatment C}	5.0199	2.2405
Var{ β_1 Line Treatment TG08}	0.9974	0.9987
Var{ β_1 Line Treatment TG50}	0.4789	0.6920
Var{ β_1 Line Generation F2}	0.8983	0.9478
Var{ β_1 Line Generation F3}	1.070	1.0345
Var{residuals}	0.1598	0.9641

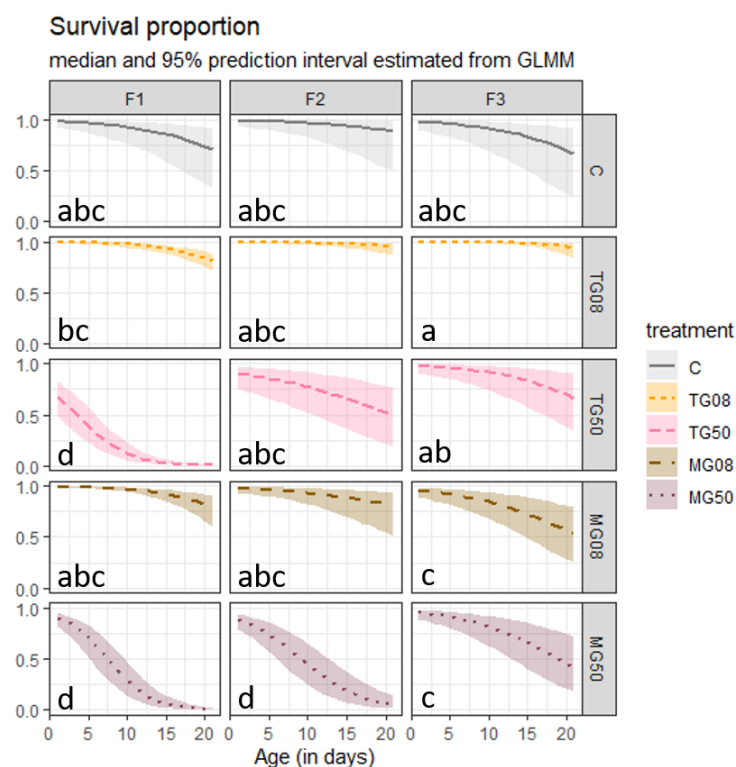


Figure S2-12. Survival proportion during 21 days according to generation and treatment, estimated from GLMMs, with merged clonal lineages. Data for controls in F3 were substituted by controls F1 (see material and methods section 2.4.1). Letters represent conditions contrasted by pairwise least-squares means comparison, with p-values adjusted with the Tukey method and a confidence level used of 0.95.

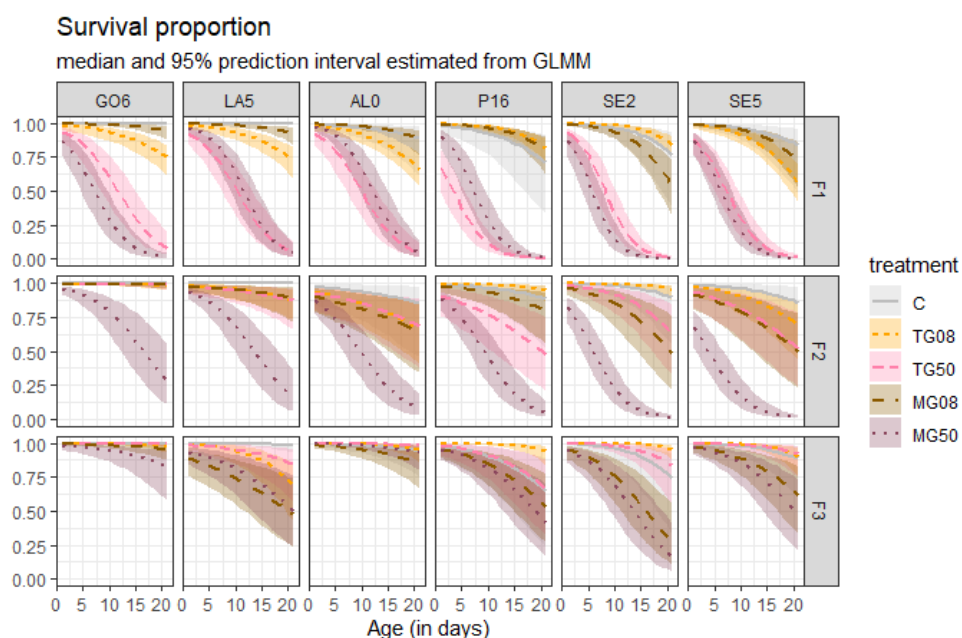


Figure S2-13. Survival proportion during 21 days according to generation, treatment, and clonal lineages, estimated from GLMMs. Data for controls in F3 were substituted by controls F1 (see material and methods section 2.4.1).

c. Effects on age at first brood, lifetime number of neonates, lifetime number of broods, and number of dead neonates

Age at first brood

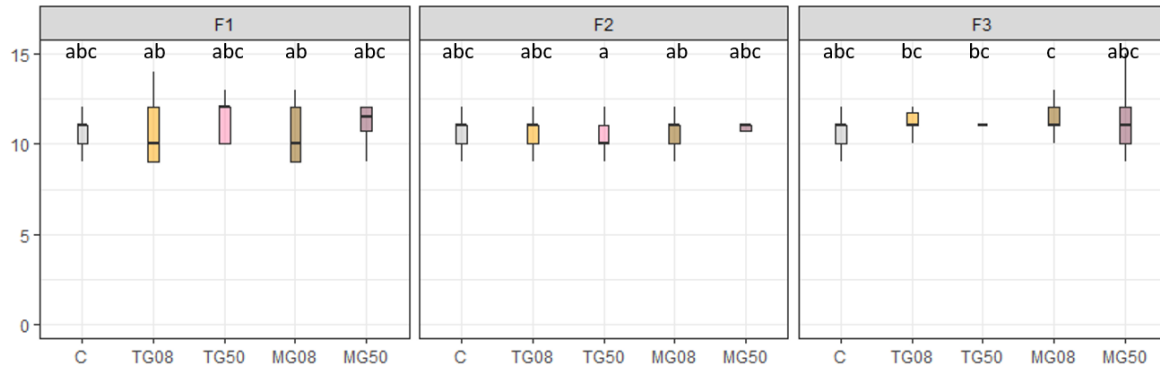


Figure S2-14. Age at first brood per female (breeding females). Data for controls in F3 were substituted by controls F1 (see material and methods). Top letters represent groups contrasted by pairwise least-squares means comparison, with p-values adjusted with the Tukey method and a confidence level used of 0.95.

Total number of neonates per dapnia

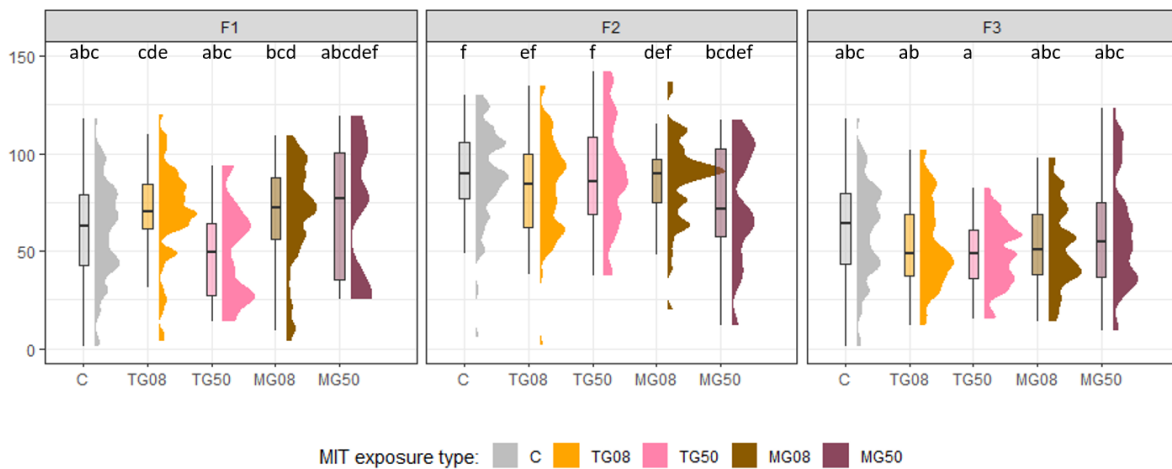


Figure S2-15. Lifetime number of neonates per female (breeding females). Data for controls in F3 were substituted by controls F1 (see material and methods). Top letters represent groups contrasted by pairwise least-squares means comparison, with p-values adjusted with the Tukey method and a confidence level used of 0.95.

Total number of broods per daphnia (who had any)

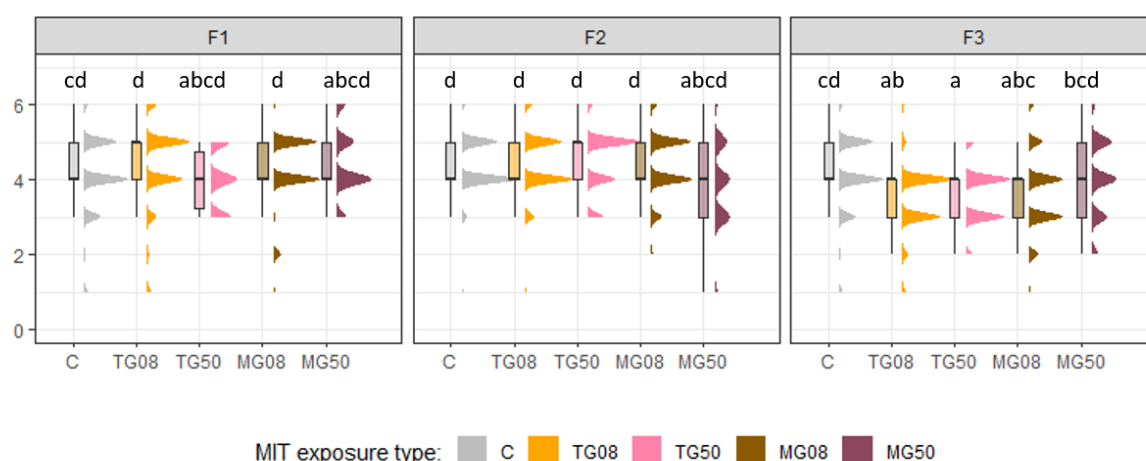


Figure S2-16. Lifetime number of broods per female (breeding females). Data for controls in F3 were substituted by controls F1 (see material and methods). Top letters represent groups contrasted by pairwise least-squares means comparison, with p-values adjusted with the Tukey method and a confidence level used of 0.95.

Total number of dead neonates per daphnia

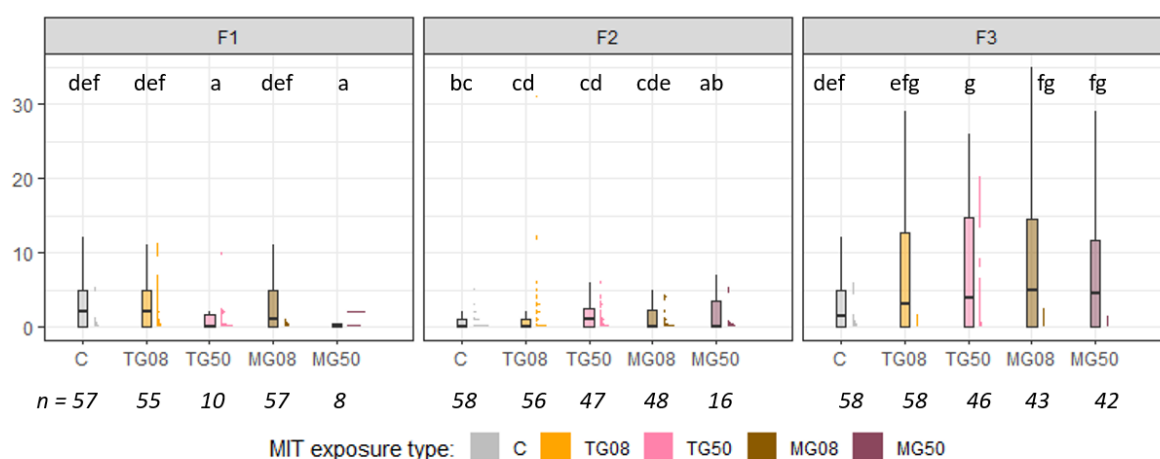


Figure S2-17. Lifetime number of dead neonates per female (breeding females, sample size indicated below each boxplot). Data for controls in F3 were substituted by controls F1 (see material and methods). Top letters represent groups contrasted by pairwise least-squares means comparison, with p-values adjusted with the Tukey method and a confidence level used of 0.95.

d. Effects on size at birth and at 21 days of age

Size of 1-day-old individuals (cm)

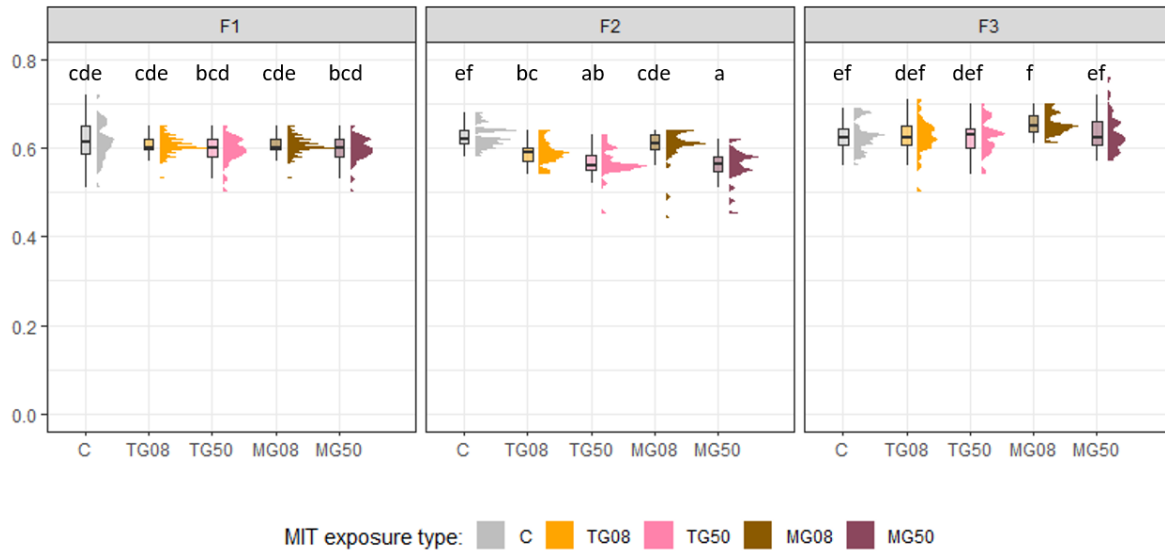


Figure S2-18. Size in cm from the centre of the eye to the base of the spine at day 1 (n=24). Top letters represent groups contrasted by pairwise least-squares means comparison, with p-values adjusted with the tukey method and a confidence level used of 0.95.

Size of 21-day-old individuals (cm)

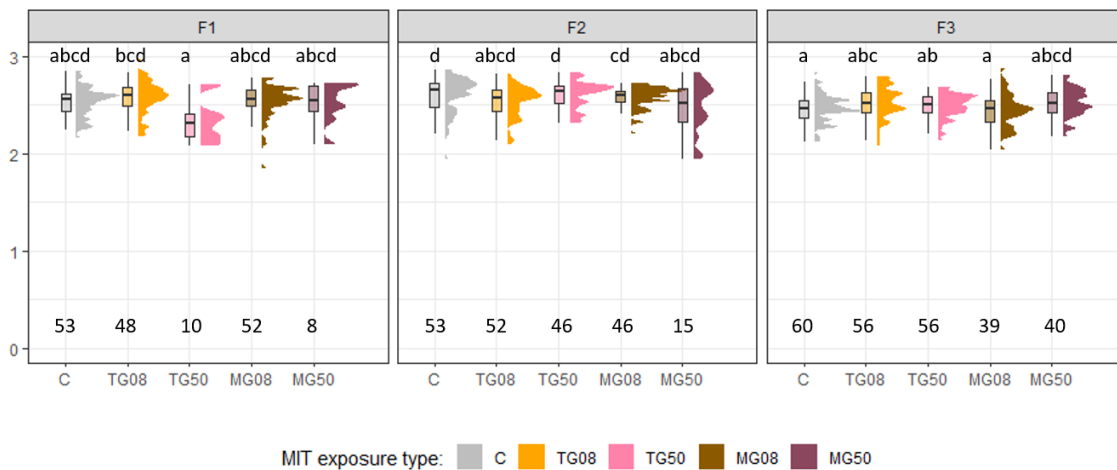


Figure S2-19. Size in cm from the centre of the eye to the base of the spine at day 21 of surviving females (sample size at the bottom of each boxplot). Top letters represent groups contrasted by pairwise least-squares means comparison, with p-values adjusted with the Tukey method and a confidence level used of 0.95.

e. Effect of lineage

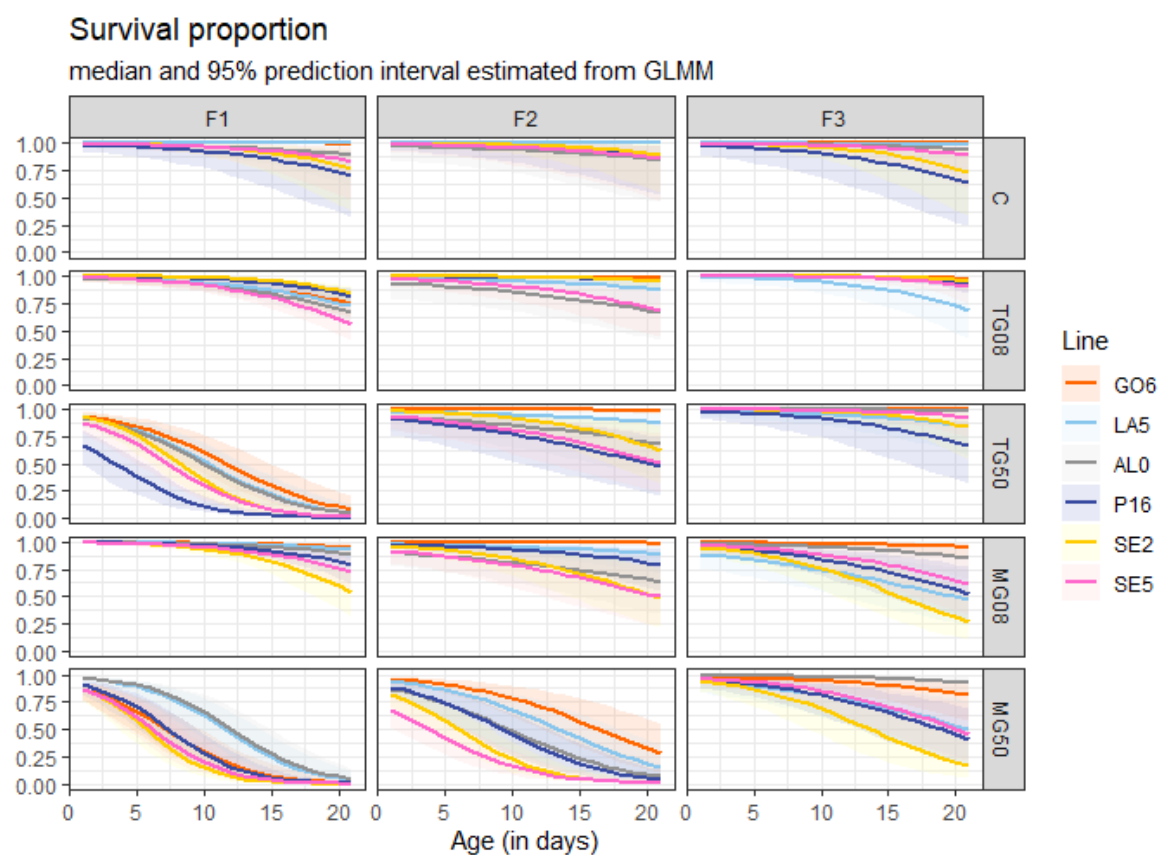


Figure S2-20. Lineages' survival response to MIT treatment during three successive generations (survival GLMM estimates: median and 95% prediction interval).

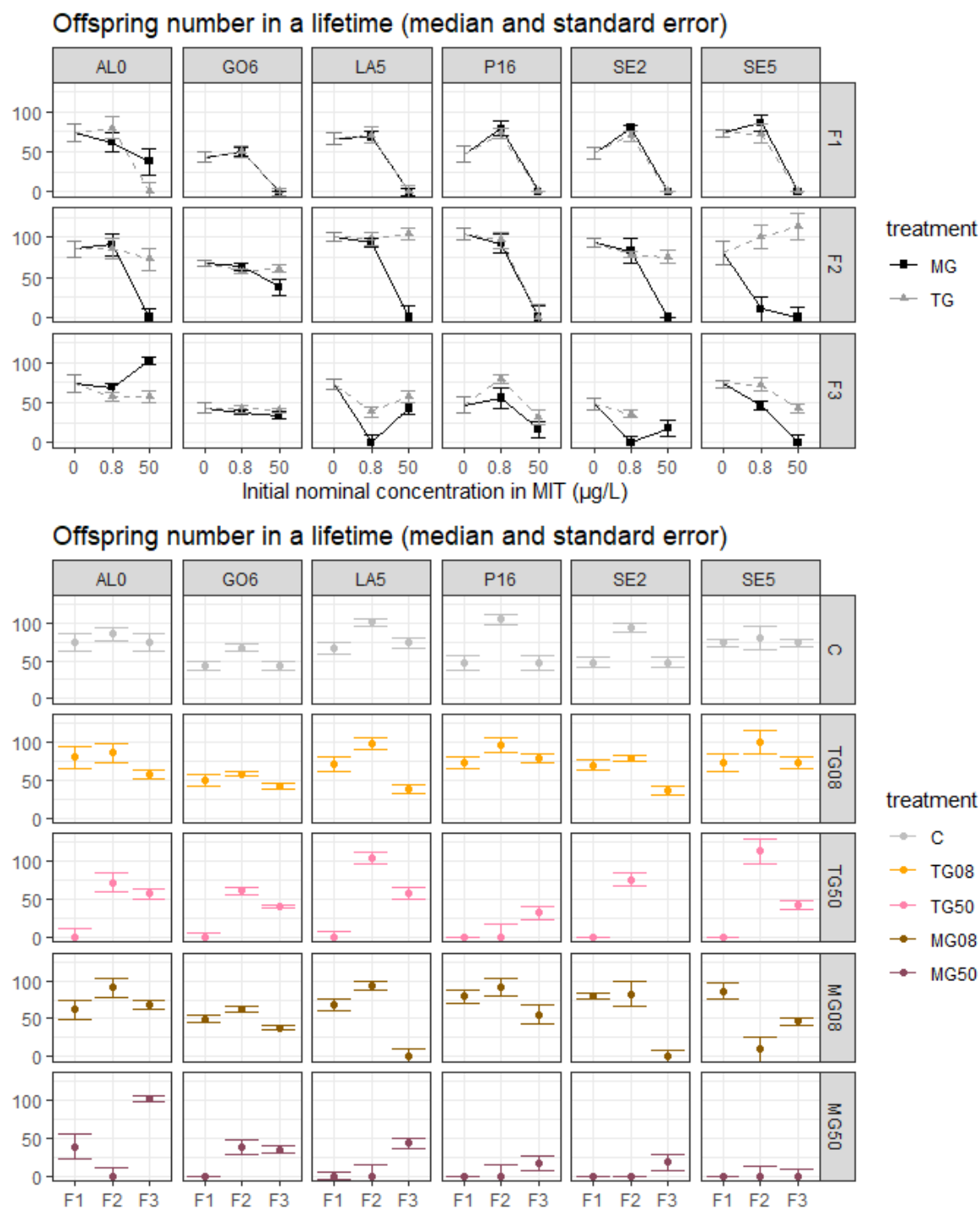


Figure S2-21. Lifetime number of offspring per female (including breeding and non-breeding females), per generation, clonal lineage, and exposure to MIT (C: controls; TG: transgenerational; MG: multigenerational; 08: maximal dose 0.8 $\mu\text{g/L}$; 50: maximal dose 50 $\mu\text{g/L}$), all lineages included (n=10).

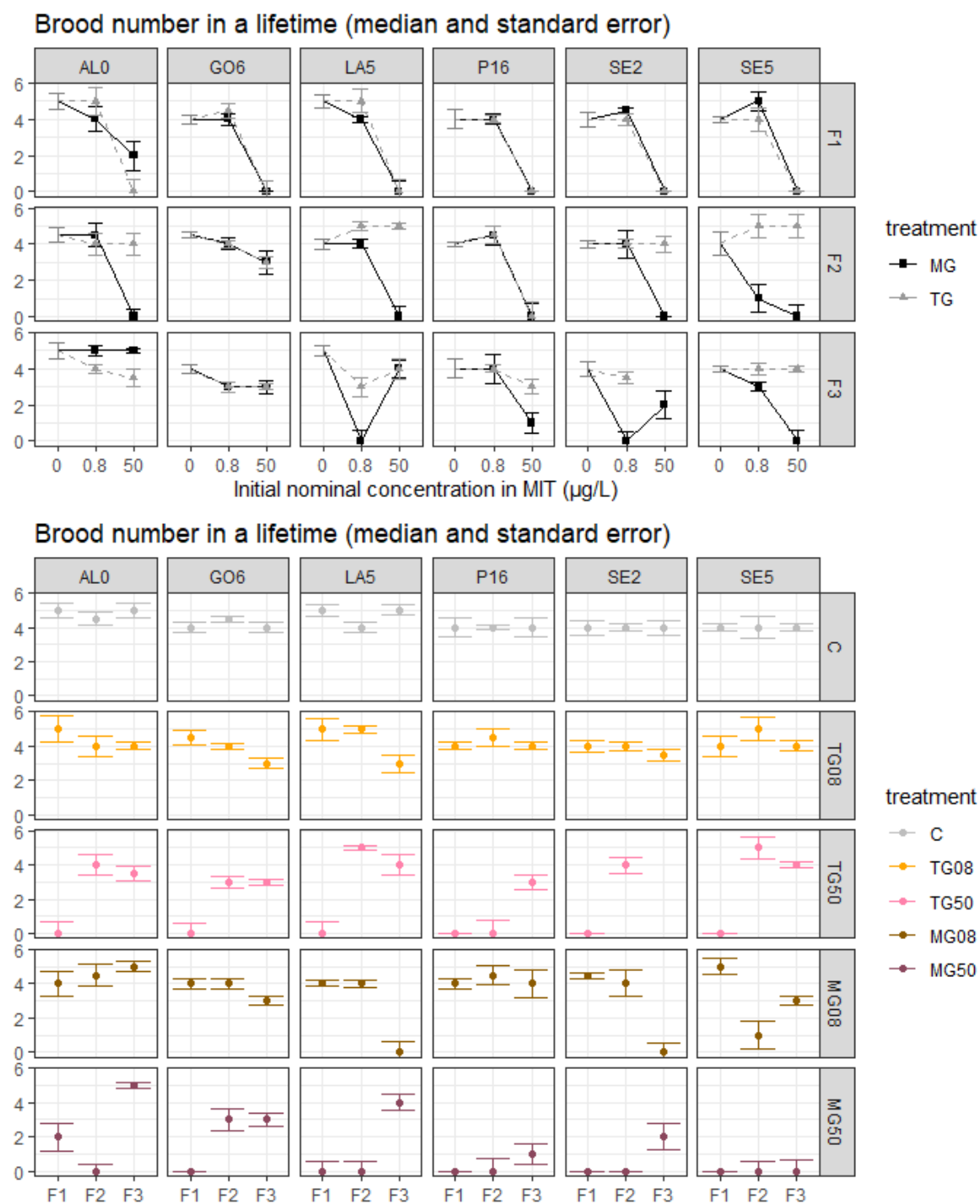


Figure S2-22. Lifetime number of broods per female (including breeding and non-breeding females), per generation, clonal lineage, and exposure to MIT (C: controls; TG: transgenerational; MG: multigenerational; 08: maximal dose 0.8 µg/L; 50: maximal dose 50 µg/L), all lineages included (n=10).

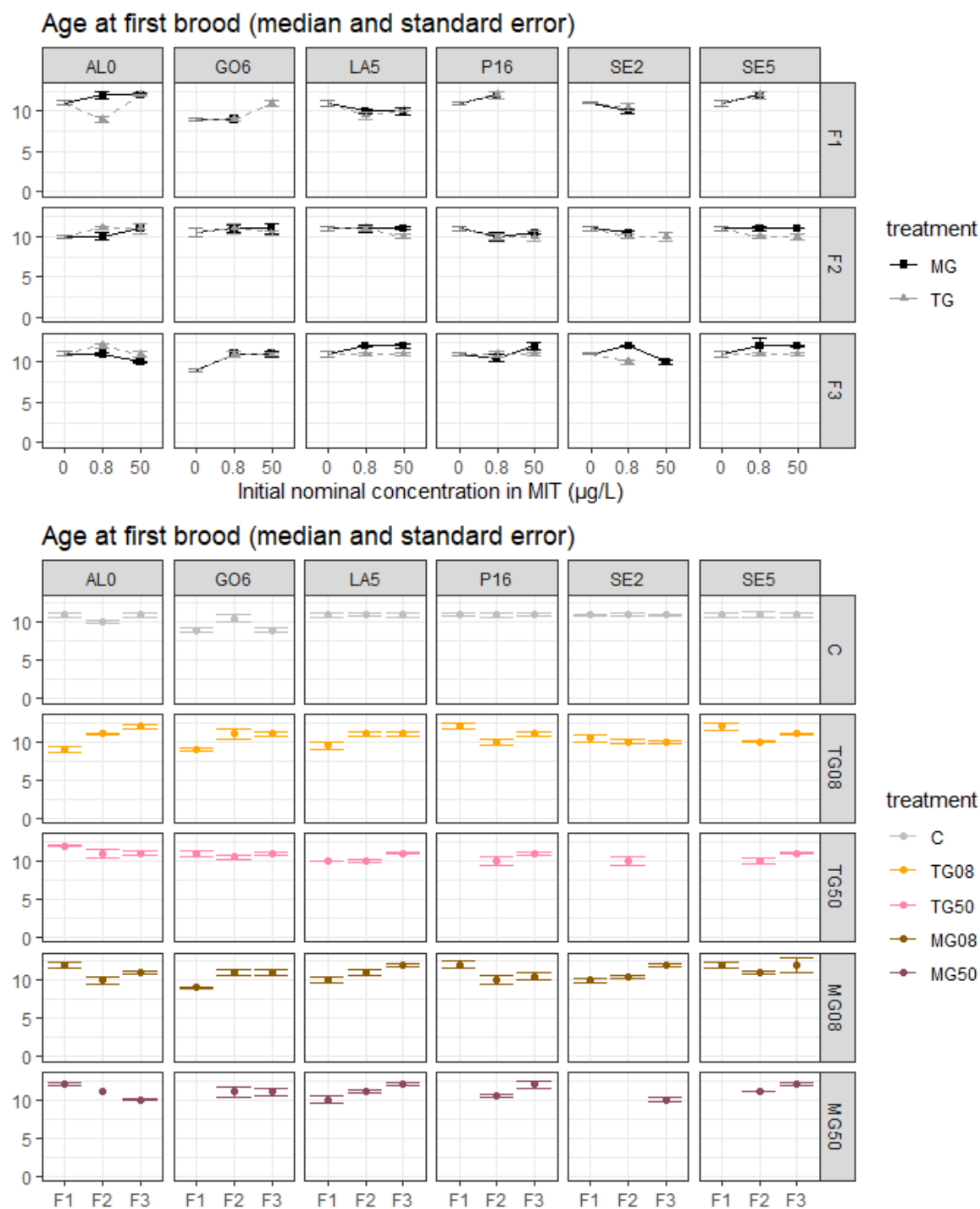


Figure S2-23. Age at first brood (including only breeding females), per generation, clonal lineage, and exposure to MIT (C: controls; TG: transgenerational; MG: multigenerational; 08: maximal dose 0.8 $\mu\text{g/L}$; 50: maximal dose 50 $\mu\text{g/L}$), all lineages included ($n=10$).

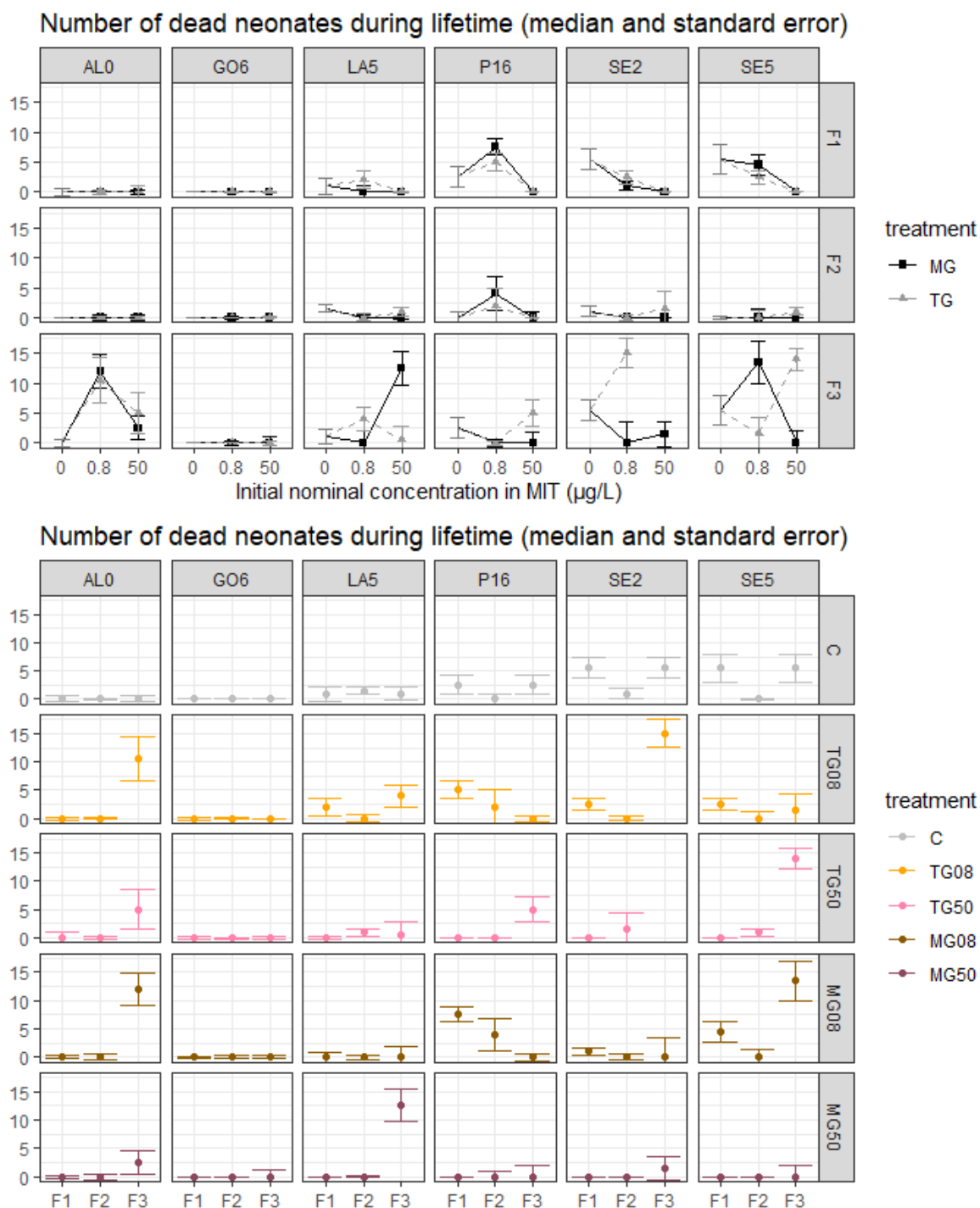


Figure S2-24. Lifetime number of dead offspring per female (including breeding and non-breeding females), per generation, clonal lineage, and exposure to MIT (C: controls; TG: transgenerational; MG: multigenerational; 08: maximal dose 0.8 $\mu\text{g/L}$; 50: maximal dose 50 $\mu\text{g/L}$), all lineages included ($n=10$).

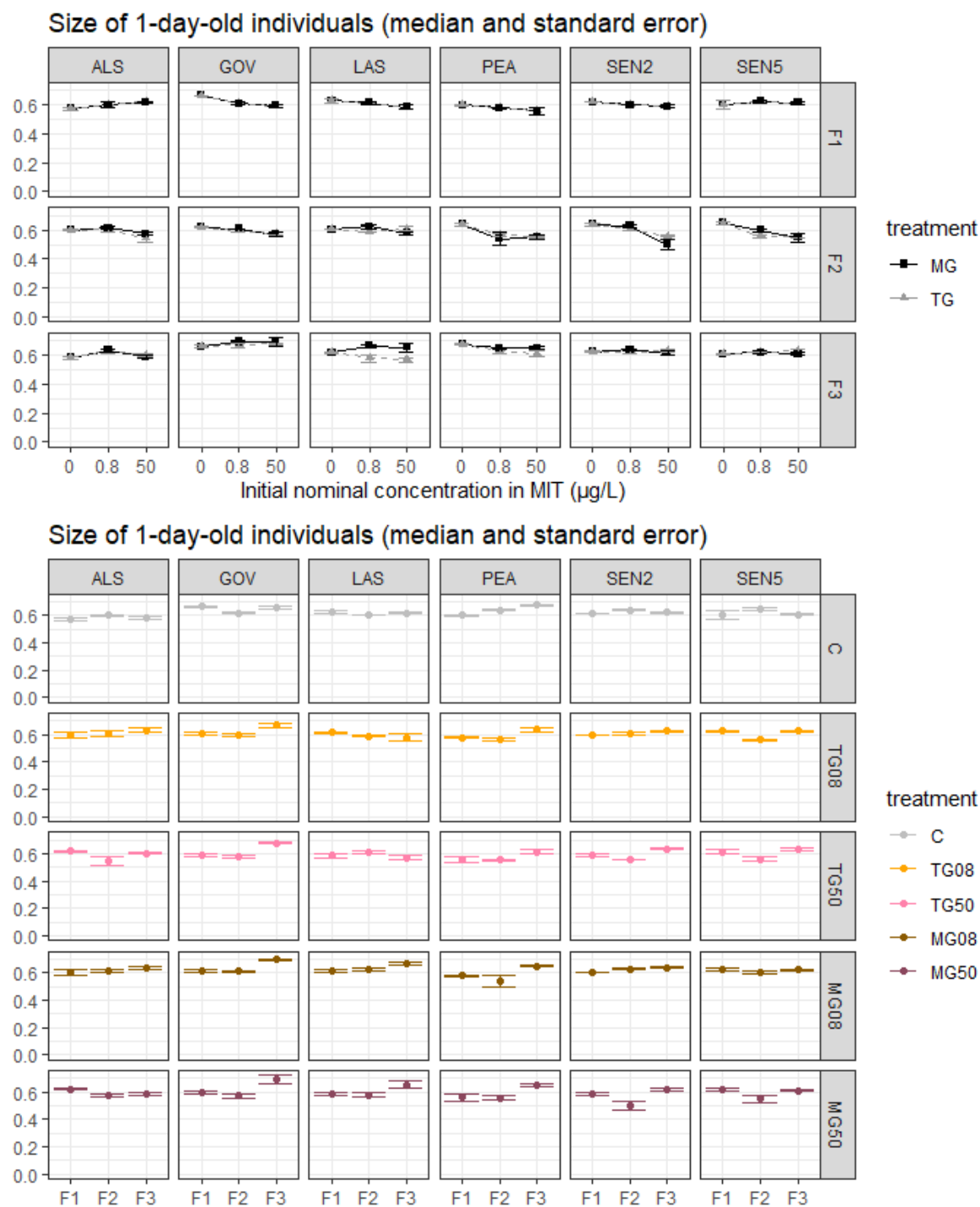


Figure S2-25. Size of neonates at day 1 ($n=4$), per generation, clonal lineage, and exposure to MIT (C: controls; TG: transgenerational; MG: multigenerational; 08: maximal dose $0.8 \mu\text{g/L}$; 50: maximal dose $50 \mu\text{g/L}$).

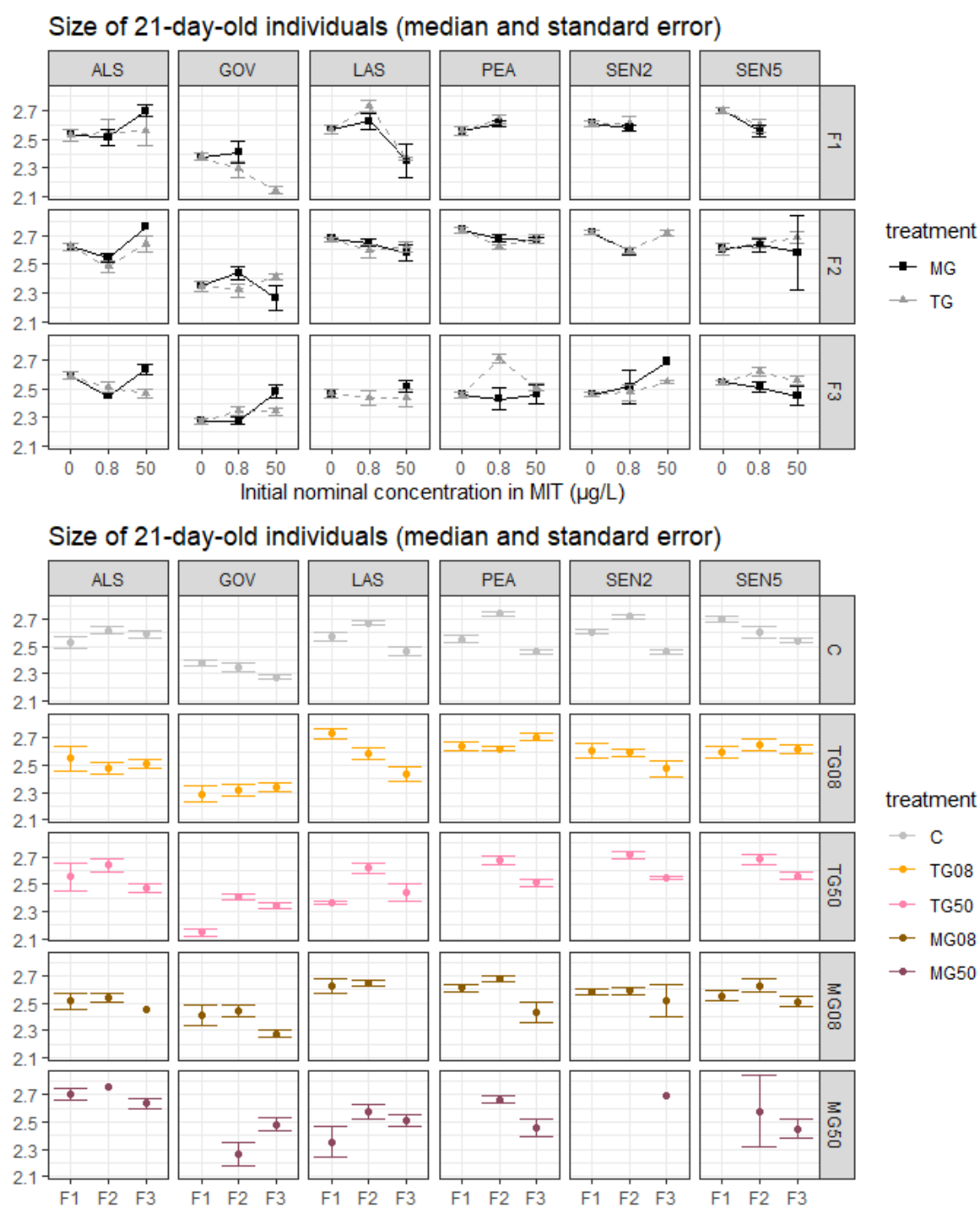


Figure S2-26. Size of surviving adults at day 21, per generation, clonal lineage, and exposure to MIT (C: controls; TG: transgenerational; MG: multigenerational; 08: maximal dose 0.8 $\mu\text{g/L}$; 50: maximal dose 50 $\mu\text{g/L}$).

SUPPLEMENTARY MATERIAL – CHAPTER 3

Sampling

Table S3-1. Sample counts per treatment and lineage. Libraries R052 and R057 (P16 MG50 F3) were incomplete, probably due to a lack of raw material, which led to sequencing failure.

Generation	Treatment	SE5	SE2	AL0	LA0	P16	G06
F1	C	3	3	3	3	3	3
	MG08	3	3	3	3	3	2
	MG50	3	3	2	4	3	3
F2	C	4	3	3	3	3	3
	MG08	3	3	3	3	3	3
	MG50	3	3	3	3	3	3
	TG08	3	3	3	3	3	3
	TG50	3	3	3	3	3	3
F3	C	3	3	3	3	3	3
	MG08	3	3	3	3	3	3
	MG50	3	3	3	3	3 (1)	3
	TG08	3	3	3	3	3	3
	TG50	3	3	2	3	3	3

Quality Control

Table S3-2. Benchmarking Universal Single-Copy Orthologs assessment of transcriptomes assemblies (1013 total BUSCO groups searched)

	SE5	SE2	AL0	LA0	P16	G06
Complete (C) (%)	72.2	98.5	79.2	74.5	76.3	76.6
C and Single-copy (%)	1.2	38.6	3.6	1.2	4.6	3.8
C and duplicated (%)	71.0	59.9	75.6	73.3	71.7	72.8
Fragmented (%)	0.0	0.8	16.3	22.0	18.4	18.3
Missing (%)	27.8	0.7	4.5	3.5	5.3	5.1

Table S3-3. Read representation of the assembly

	SE5	SE2	AL0	LA0	P16	G06
Nb reads	1,800,865,3	1,925,976,2	1,699,582,2	1,908,364,4	1,742,102,3	1,846,239,5
	51	19	87	21	79	53
Paired (%)	100	100	100	100	100	100
Aligned concordantly 0 times (%)	40.48	27.38	38.02	40.00	41.63	43.97
Aligned concordantly 1 time (%)	10.21	24.37	11.78	9.36	9.53	7.08
Aligned concordantly > 1 times (%)	49.32	48.25	50.20	50.64	48.83	48.95
Overall alignment rate (%)	78.74	83.83	80.03	78.72	77.34	75.47

Table S3-4. Trinity transcriptomes contig statistics

	SE5	SE2	ALO	LAO	P16	G06
Total trinity transcripts	93557	65950	88230	92783	92544	95908
Total trinity 'genes'	229034	180692	218507	235011	232083	247135
Percent GC	42.06%	45.23%	41.99%	42.11%	42.05%	42.37%
Stats based on all transcript contigs						
Median contig length	697	981	765	743	733	699
Average contig	1035.88	1380.42	1176.56	1119.91	1084.09	1055.71
Total assembled bases	237251397	249430436	257086515	263191043	251598823	260901836
Contig N50	1556	2132	1854	1725	1635	1593
Stats based on only the longest isoform per gene						
Median contig length	464	863	481	474	476	457
Average contig	774.75	1303.91	841.69	818.37	804.83	778.64
Total assembled bases	72482892	85992879	74262101	75930802	74482437	74677466
Contig N50	1185	2172	1366	1302	1257	1208

Differential Expression Analysis

Table S3-5. MIT-induced differential expression across lines and generations: number of DEGs (padj <0.01, logFoldChange>1). * P16-MG50-F3 is given only as indicative value, due to the lack of replication in this modality.

		ALO	GO6	LAO	P16	SE2	SE5
MG08	F1	1045	288	340	469	2887	296
	F2	749	655	761	463	479	905
	F3	435	830	635	643	459	905
MG50	F1	696	3011	1100	766	489	1033
	F2	530	673	639	630	486	710
	F3	315	1933	512	819*	523	497
TG08	F2	432	642	784	616	356	702
	F3	407	1513	751	639	504	928
TG50	F2	472	1125	932	572	1171	899
	F3	288	1500	765	540	406	842

Table S3-6. Number of DETs per lineage.

Lineage	Total number of Differentially Expressed TRINITY transcripts (TRI-DETs)	Number of TRI-DETs annotated against Dpulex Uniprot DB (AnTRI-DETs)	Number of different annotations (Dpulex Uniprot DB)	Number of AnTRI-DETs with a least 1 GO term	Number of AnTRI-DETs with a least 1 KEGG term
AL0	2612	1895 (73%)	1563	1038 (55%)	1413 (75%)
GO6	4327	3301 (76%)	2406	1531 (46%)	699 (21%)
LA0	2410	1793 (74%)	1383	822 (46%)	410 (23%)
P16	2518	1902 (76%)	1475	982 (52%)	512 (27%)
SE2	4481	3954 (88%)	2965	2251 (57%)	1414 (36%)
SE5	3069	2339 (76%)	1778	1197 (51%)	662 (28%)

Table S3-6. GO terms of DEGs best hits against *Daphnia pulex* proteome (padj <0.01) can be found at https://github.com/mwagnerdeyries/MIT_toxicity_Dpulex_thesis

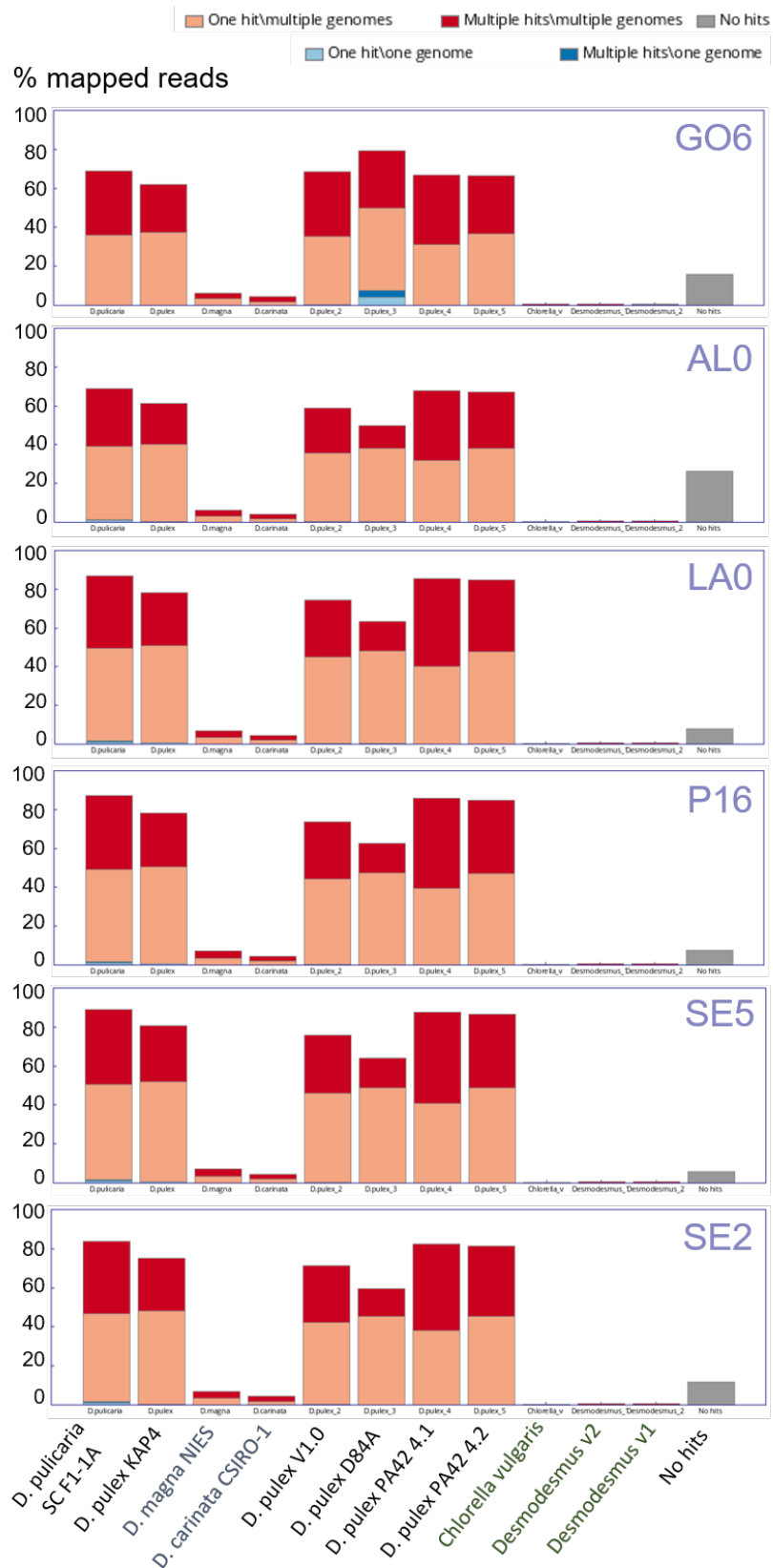


Figure S3-1. Percentage of DNA reads mapped on a selection of reference genomes (with strains indicated) for the six lineages used in the chronic exposure to MIT (FastQ Screen v 0.14.0). Most reads are mostly mapped once (light red) or several times (dark red) on the several genomes of *Daphnia pulex* and *D. pulicaria*, and a few of them are mapped once (light blue) or several times (dark blue) on only one of the reference genomes.

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Titre : Toxicité de la méthylisothiazolinone sur l'espèce clé de voûte des écosystèmes d'eau douce *Daphnia pulex*

Mots clés : Multigénération, variabilité intraspécifique, écotoxicologie évolutive, transcriptome

Résumé : Les isothiazolinones sont des molécules organiques utilisées comme biocides à large spectre dans une grande variété d'applications industrielles et de consommation. Bien que leur présence dans les sols et les eaux douces ait été détectée, les rapports des fabricants ont jugé les quantités concernées inoffensives pour l'écosystème. Toutefois, compte tenu des quantités libérées dans l'environnement depuis des décennies, des inquiétudes ont été exprimées quant à leur impact sur les processus d'évolution des espèces non ciblées, qui ne sont pas examinés dans des conditions de tests de toxicité standard. Ce travail vise à étudier en particulier les effets de la plus inoffensive de ces molécules, la méthylisothiazolinone (MIT). Sa toxicité aiguë a été évaluée sur *Daphnia pulex*, un invertébré aquatique emblématique en écotoxicologie et en biologie évolutive.

Ces tests à court terme ont révélé une grande variabilité intraspécifique dans la tolérance, ce qui a également été vérifié dans une expérience à long terme utilisant de faibles concentrations de MIT. En outre, l'exposition à la MIT a eu des effets létaux et sublétaux sur la fitness des daphnies, avec une évolution sur quatre générations dans le cas d'une exposition multigénérationnelle continue, mais pas dans le cas d'une exposition parentale ou grand-parentale. Ces effets ont été étudiés plus en détail par analyse d'expression différentielle à partir de données globales d'ARNseq. La variabilité intraspécifique et les changements dans les réponses au sein d'une espèce appellent à l'inclusion des processus évolutifs dans les évaluations des risques et au réexamen des interprétations des tests d'écotoxicité standards.

Title: Methylisothiazolinone toxicity on the freshwater keystone species *Daphnia pulex*.

Keywords: Multigenerational, Intraspecific variability, Evolutionary ecotoxicology, Transcriptome

Abstract: Isothiazolinones are a family of organic molecules used as broad-spectrum biocides in a wide variety of consumer and industrial applications. Although their presence in the environment has been detected, especially in soils and freshwater, reports by the manufacturers deemed the quantities involved harmless for the ecosystem. However, given the daily amounts of it released in the environment over decades, concerns were raised about their impact on non-target species evolutionary processes, which cannot be examined under standard ecotoxicity test conditions. This work aims to provide some answers by studying the effects of the most innocuous of these molecules, methylisothiazolinone (MIT). MIT acute toxicity was assessed on *Daphnia pulex*, an iconic ecotoxicology and evolutionary model species of aquatic invertebrates.

These short-term tests revealed a great intraspecific variability in the tolerance to MIT contamination, which was also verified in a long-term experiment using low concentration. Moreover, MIT exposure caused lethal and sublethal effects on daphnia fitness, with developments over four generations in the case of a continuous multigenerational exposure, but not in the case of parental or grandparental exposure. These effects were further investigated with differential expression analysis from global RNAseq data. Both intraspecific variability and multigenerational change in the responses within one species call for the inclusion of evolutionary processes in risk assessments and the reconsideration of standard ecotoxicity tests interpretations.